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pH sensitive liposomes for the treatment of rheumatoid arthritis

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Abstract

Rheumatoid arthritis (RA) is a chronic systemic inflammatory and autoimmune disease mainly characterized by the progressive inflammation of the synovial tissue of the body joints, destruction of cartilage and further bone erosion. Currently available treatment options include non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids (GCs) and disease modifying anti-rheumatic drugs (DMARDs), either used as monotherapy or in combination therapy. However, all of these therapeutic strategies are associated with severe side effects resultant from limited selectivity and widespread biodistribution of drug molecules into non-target tissues. In order to overcome the drawbacks of conventional therapy, the aim of the following dissertation is to design pH-sensitive liposomes as suitable drug delivery nanosystems for the treatment of RA. Although these liposomes are stable at physiological pH, they undergo rapid liposomal destabilization under mildly acidic conditions as those presented in endosomes of target cells. Thus, promising to improve the therapeutic efficiency of a GC drug - Prednisolone Disodium Phosphate (PDP) -, due to their ability to mediate an intracellular, specific and controlled release of the drug molecules, while limiting adverse off-target unwanted effects. In this sense, pH-sensitive liposomes modified (or not) with specific targeting ligands, as the polyethylene glycol-folic acid (PEG-FA) or the hyaluronic acid (HA), were developed, to enhance the selective and efficient delivery of loaded PDP into target synovial macrophages and fibroblast. Furthermore, the *in vitro* therapeutic performance of the designed pH-sensitive liposomes was evaluated, through the optimization of its lipid composition, physicochemical characteristics, drug release studies mimicking both biological conditions at pH 7.4 and pH 5.0, cellular studies and, as well as, the liposomal stability during storage. The selectivity and stability of the proposed pH-sensitive liposomes increases the bioavailability of the PDP at the site of inflammation, once the liposomes specifically internalize into the target cells where they trigger the release of drug and thereby enhance the therapeutic effect, reducing the number of dosages and minimizing the known deleterious side effects of PDP.

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Resumo

A artrite reumatoide (AR) é uma doença inflamatória autoimune sistêmica e crônica, principalmente caracterizada pela inflamação progressiva do tecido sinovial das articulações do corpo, destruição da cartilagem e posterior erosão do osso. As opções de tratamento atualmente disponíveis incluem fármacos anti-inflamatórios não-esteroides, glucocorticoides (GCs) e fármacos antirreumáticos modificadores da doença, seja usados como monoterapia ou em terapia combinada. No entanto, todas estas estratégias terapêuticas estão associadas com efeitos secundários graves resultantes de seletividade limitada e biodistribuição generalizada das moléculas de fármaco nos tecidos não-alvo. A fim de superar as desvantagens da terapia convencional, o objetivo da presente dissertação é desenhar lipossomas sensíveis ao pH como nanossistemas de entrega de fármacos adequados para o tratamento da AR. Embora estes lipossomas sejam estáveis a pH fisiológico, são submetidos à rápida desestabilização lipossomal sob condições ácidas como as apresentadas nos endossomas das células alvo. Assim, a promessa de melhorar a eficácia terapêutica de um fármaco GC - prednisolona fosfato disódico (PDP) -, devido à sua capacidade para mediar a libertação intracelular específica e controlada das moléculas de fármaco, limitando ao mesmo tempo efeitos adversos indesejados fora do alvo. Neste sentido, foram desenvolvidos lipossomas sensíveis ao pH modificados (ou não) com ligandos específicos, como o polietilenoglicol-ácido fólico (PEG-FA) ou o ácido hialurônico (HA), para aumentar a libertação seletiva e eficiente de PDP nos macrófagos e fibroblastos sinoviais alvo. Além disso, o desempenho terapêutico *in vitro* dos lipossomas sensíveis ao pH concebidos foi avaliado, através da otimização da composição lipídica, de características físico-químicas, de estudos de libertação de fármaco que mimetizam ambas as condições biológicas a pH 7.4 e pH 5.0, de estudos celulares, bem como, a estabilidade lipossomal durante o armazenamento. A seletividade e estabilidade dos lipossomas sensíveis ao pH propostos aumenta a biodisponibilidade do PDP no local da inflamação, uma vez que os lipossomas internalizam especificamente nas células-alvo, onde provocam a libertação do fármaco e, assim, melhoraram o efeito terapêutico, reduzindo o número de dosagens e minimizando os efeitos colaterais deletérios da PDP.

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*“ Não vos inquieteis, pois, com o dia de amanhã: o amanhã se ocupará de si.
A cada dia basta o seu trabalho! “*

*Sermão da Montanha
Segundo S.Mateus
5,1-7,29*

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Abbreviations and Symbols

AA	arachidonic acid
CHEMS	cholesteryl hemisuccinate
COX	cyclooxygenase
DCC	dicyclohexylcarbodiimide
DCU	dicyclohexylurea
DLS	Dynamic Light Scattering
DMARD	disease modifying anti-rheumatic drug
DMEM	Dulbecco's Modified Eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPPE	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine
DSPE	distearoylphosphatidylethanolamine
EDC	ethyl dimethyl-aminopropyl-carbodiimide
EE	encapsulation efficiency
ELS	Electrophoretic Light Scattering
EPR	enhanced permeation and retention
EULAR	European League Against Rheumatism
FA	folic acid
FBS	fetal bovine serum
FR	folate receptor
GC	glucocorticoid
HA	hyaluronic acid
H _{II}	inverted hexagonal phase
IFN	interferon
IL	interleukin
LC	loading capacity
LDH	lactate dehydrogenase
LO	lipoxygenase
LUV	large unilamellar vesicles
L _C , L _β , L _α	crystalline lamellar, lamellar gel, lamellar liquid-crystalline
MLV	multilamellar vesicles

MMPs	matrix metalloproteinases
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide
MTX	methotrexate
NBD-DPPE	1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)
NHS	N-Hydroxysuccinimide ester
NSAID	non-steroidal anti-inflammatory drug
PALS	Phase Analysis Light Scattering
PBS	phosphate-buffered saline
PDP	prednisolone disodium phosphate
PE	phosphatidylethanolamine
PEG	polyethylene glycol
PLA ₂	phospholipase A ₂
QELS	quasi-elastic light scattering
RA	rheumatoid arthritis
RES	reticuloendothelial system
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
SD	standard deviation
SUV	small unilamellar vesicles
TEA	triethylamine
TEM	transmission electron microscopy
T _m	phase transition temperature
TNF- α	tumor necrosis factor- α
UV/Vis	Ultraviolet-visible
VEGF	vascular endothelial growth factor
λ	wavelength

Chapter 1

Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is a chronic systemic inflammatory autoimmune disease that afflicts approximately 1-2 % of the world population. [1, 2] The degeneration of the joints and inflammation in the synovium is owed to the chronic nature of this arthritic disease and its ability to affect other organs makes it a systemic disease. However, there is still some uncertainty about the exact cause for the immune response originated from RA in which the immune system attacks the body's own cells and causes inflammation.

RA may affect many tissues and organs, but mainly attacks synovial joints of the bone, and preferentially targets the small joints with frequent movement such as wrists, neck, hands and feet. As well, its manifestation in weight bearing joints such as hips, knees, spine and ankles develops later. [3, 4] The progressive inflammation of the synovial tissue of the multiple joints of the body, greatly compromise the life quality of patients who suffer from it, due to the destruction of cartilage and bone of the affected joints. [2, 3, 5]

1.1 Pathophysiology

The pathophysiology of RA is mainly characterized by erosion of the cartilage and osseous tissue, which leads to chronic synovial hypertrophy of the joint adjacent to the inflammatory reaction. At the early stage, swelling and pain in the joint is developed, which triggers characteristic cartilage deformation and joint stiffness as the arthritis progresses. Hence, after prolonged inflammation, RA can lead to cartilage degradation, bone erosion, functional impairment and eventual joint disability. [3-5] These

destructive joint changes are a hallmark of RA and serve as a measure of disease severity. [3]

Although the exact origin of the RA onset remains unknown, the inflammation triggered by RA is initiated by a self-immunological response. In RA inflamed joints (Figure 1), synovial membranes become thicker and the synovial space undergoes a sustained inflammation mediated by the release of proinflammatory cytokines and matrix metalloproteinases (MMPs), which followed by the proliferation of numerous inflammatory-immune cell types, including T cells, B cells, macrophages, neutrophils and synoviocytes (synovial fibroblasts). Then, the inflamed synovial membrane progresses into joint destruction by eroding the cartilage and bone tissue. [6-8] On the other hand, a normal joint allows movement without any pain or discomfort due to the synovium (joint capsule lining) producing synovial fluid which lubricates the movement of the smooth cartilage at the ends of the bone. [9]

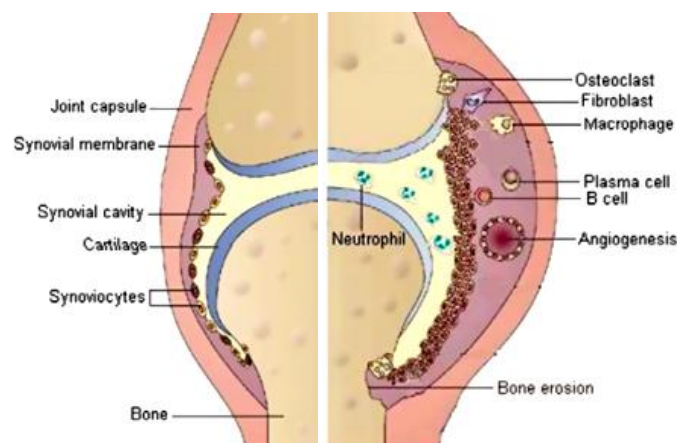


Figure 1 Normal joint (left) and RA joint presenting the pannus tissue with numerous types of cells (right). Adapted from [9].

Thus, inflammatory immune cells are believed to be the key regulators that play an important role in pathogenesis of disease (Figure 2). [1] The B cells produce molecules which then mediate the immunologic process by increasing the permeability of the vessels responsible for irrigation of the inflamed tissues – vasodilatation. [1, 2, 10] Likewise, T cells prominent in RA synovium actively contribute to the inflammatory response, through a process called chemotaxis. The activated T cells in the synovium produce interleukin-2 (IL-2) and interferon- γ (IFN- γ) cytokines which attract inflammatory cells like macrophages and leucocytes that perpetuate the inflammation and joint destruction. [1, 2, 10]

The activation of these inflammatory cells overproduce cytokines, including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6), which stimulate the proliferation of synoviocytes. [1, 2, 6] Especially, both TNF- α and IL-1 β induce activated synoviocytes to release tissue degrading MMPs and to stimulate the differentiation and proliferation of osteoclasts responsible for joint destruction. [1, 7, 11] Moreover, synovial fibroblast are considered to be responsible for the progression of the disease from one arthritic joint to other, unaffected joints, a role which bears resemblance to that of metastatic tumor cells in cancer. [12] Similarly, the activation of vascular endothelial growth factor (VEGF) by synovial fibroblasts induces angiogenesis, comparable to that occurs during tumor growth, hence perpetuates the inflammation by recruiting more inflammatory leukocytes. [13, 14] As a result, the growth of the pannus into the joint cartilage induces a state of relative hypoxia that further promotes angiogenesis. [1]

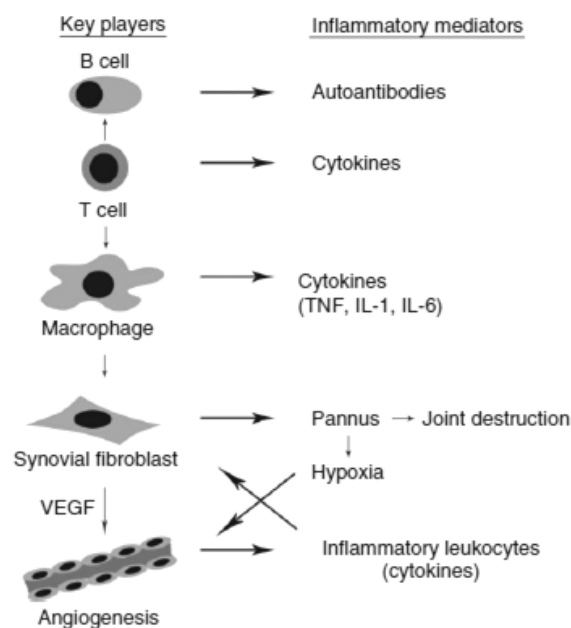


Figure 2 Pathogenesis of RA. Adapted from [1].

Furthermore, the inflammatory process and adjacent immune response to the joint injury consists in a complex set of cellular changes involving the activation of enzymes and secretion of multiple chemical mediators. Among the chemical mediators involved in the inflammatory response, those resulting from prostaglandin synthesis should be emphasized by their importance as potent mediators of inflammation and tissue destruction. The synthesis of prostaglandins is activated in response to inflammatory stimuli, by the enzyme phospholipase A₂ (PLA₂), which hydrolyses membrane

phospholipids with the consequent release of free fatty acids, namely arachidonic acid, a polyunsaturated fatty acid. The arachidonic acid (AA) can be metabolized by two different pathways. One depends on the action of an enzyme complex known as cyclooxygenase (COX-1 and COX-2), that induces the production of prostaglandins. The other involves the activity of the enzyme 5-lipoxygenase (5-LO) which converts AA to leukotrienes. [10]

Thus, both the enzymatic and osteoclastic destruction of the arthritic joint leads to joint deformation and loss of function, and to pain and morbidity for patients suffering from RA. Although not regarded as a lethal disease, RA reduces the mean life expectancy of patients with 5–10 years, depending on disease severity. [15]

1.2 Current therapeutic treatment

Treatment for RA has significantly improved in recent years, evolving from a strategy of providing symptomatic relief, to implementation of therapeutic regimens that impact disease activity and lately to slow or arrest structural joint damage. [16] During the first half of the 20th century the progression of RA from symptom onset to significant disability was often inevitable. [17] Nowadays, with the medications available is possible to slow down disease progression preventing irreversible joint damage. Joint replacement surgery is not always the final outcome, and RA patients may live comfortable and productive lives on medical therapy. [18] Presently is generally known that there is a short time frame of opportunity, immediately after disease onset, for effective therapy and subsequently to prevent long-term structural damage with reduction of functional impairment. Although management of RA treatment requires a multidimensional approach, currently available drug therapy for RA has made remission an achievable treatment goal. [2, 19] Clinically, the requirements for the management of RA involve the preservation of the joint mobility, relieve pain and minimize inflammation, and consequently, reduction of joint degeneration, deformities and loss of function. [4]

Conventional drugs used in treatment of RA are mainly classified as first line agents and second line agents. [20] First line agents mainly involve non-steroidal anti-inflammatory drugs (NSAIDs) and steroids such as glucocorticoids (GCs), characterized by a rapid effect and promote a rapid suppression of inflammatory symptoms, relieving pain, swelling and stiffness manifested in RA. However these

agents do not prevent further tissue damage of joints. [1, 20] On the other hand, second line agents mainly include synthetic and biological disease modifying anti-rheumatic drugs (sDMARDs and bDMARDs, respectively), that exert a prolonged action, modifying the course of the disease, leading to a more effective prevention of joint destruction. [9, 20] Further, the conventional therapies available in the clinical management of RA will be briefly described.

Currently, the strategy implemented in the treatment of RA is an early intervention in the course of the disease to delay the onset of joint damage. A combination of first and second line drugs is used to enhance the possibility of eliminating the initial inflammatory process and, consequently, preventing joint damage. [21] Available treatment options for RA aim at symptomatic pain relief using NSAIDs on the one hand, and on the other slowing down disease progression/activity with DMARDs and GCs to achieve remission. [2, 5] However, GCs and DMARDs are given if the treatment with NSAIDs proves to be ineffective. [2] Still, most treatments still require frequent and long-term administration by conventional routes (oral and intra-articular injections), leading to undesired systemic side effects and extra-synovial accumulation of drugs.

1.2.1 Non-steroidal anti-inflammatory drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ibuprofen, naproxen and celecoxib have been extensively used for the treatment of RA due to their quick onset of analgesic and anti-inflammatory effects for symptomatic relief. [2, 22]

NSAIDs do not alter the disease progress and articular damage, instead they act as inhibitors of the enzymes COX-1 and COX-2, which are responsible for synthesis of prostaglandins, that play a key role in inducing pain and inflammation. [5, 23]

However, NSAIDs display a short half-life after oral administration, demanding frequent and high dosing to achieve a full therapeutic efficiency in RA treatment, thus increasing the gastrointestinal side effects. [2, 5] Moreover the side effects of NSAIDs include peripheral oedema, platelet inhibition, renal necrosis, nephrotic syndrome and hepatic injury, among others. [22, 23]

1.2.2 Glucocorticoids

Glucocorticoids (GCs) such as prednisolone, methylprednisolone, dexamethasone and budesonide can be highly effective as first-line drugs in RA by controlling the inflammatory process in RA and suppressing the progression of the immunologic response. [6]

The mechanism of action of GCs aims to inhibit leukocyte infiltration at the site of inflammation either by inciting mediators of inflammatory process or by suppressing the immune responses. The anti-inflammatory actions of GCs drugs are thought to come from the inhibition of PLA₂ enzyme, which controls the biosynthesis of strong chemical mediators of inflammation, such as prostaglandins and leukotrienes. Additionally, GCs moderate inflammatory reaction by limiting the vasodilatation phenomenon through a reduction of the release of vasoactive kinins and, therefore, restricts the accumulation of inflammatory leukocytes. [3, 20]

Furthermore, GCs drugs can activate and influence biochemical behavior of most cells, including of immune cells. GCs bind to a glucocorticoid receptor agonist that passively allows the diffusion across the cell membrane. Upon binding, the corticoid receptor-ligand complex interacts with glucocorticoid response elements in the promoter region of anti-inflammatory genes, inducing variations in the expression of specific cytokine genes (e.g. IL-2, IL-1 and TNF- α). [3, 20] Thus, GCs drugs cause inhibition of inflammatory response by interfering with immunological agents and thus prevents inflammation.

Although GCs are most potent anti-inflammatory drugs and exhibit rapid onset of action, long term use and high doses of steroids is associated with severe side effects, including impaired wound healing, skin atrophy, hyperglycaemia, hypertension, weight gain, increased risks of cardiovascular diseases, osteoporosis, muscle atrophy, cataract, glaucoma, peptic ulcer, manifestation of latent diabetes, and ultimately premature mortality. [1, 6, 22] In fact, all patients on GC therapy usually receive appropriate treatment to minimize the risk of steroid-induced osteoporosis (such as, receive supplemental calcium and vitamin D) and careful attention to the development of other toxicities. [3, 22, 24] Hence, GCs are mainly used as a therapeutic additive in low doses or as a combine therapy aimed at controlling symptoms during periods when disease activity is high. [22] Thus, GCs drugs represent an affordable class of anti-inflammatory agents that are widely used in active RA as co-therapy with

DMARDs. [1] Being, therefore, useful either as temporary therapy, until the response to DMARDs is achieved, and as chronic therapy in severe RA that is not well controlled with use of DMARDs. [9] Studies have suggested that low-dose GCs may have disease modifying effects in RA. [25, 26]

1.2.3 Disease modifying anti-rheumatic drugs

A new nomenclature for disease modifying anti-rheumatic drugs (DMARDs) has been recently proposed where synthetic DMARDs were divided into conventional synthetic and targeted synthetic DMARDs (csDMARDs and tsDMARDs, respectively) and biologic DMARDs into biological original and biosimilar DMARDs (boDMARDs and bsDMARDs, respectively). tsDMARDs would then include only those drugs specifically developed to target a particular molecular structure (e.g. tofacitinib, fostamatinib or agents not focused primarily on rheumatic diseases, such as imatinib or ibrutinib), while csDMARDs would comprise the traditional drugs (such as methotrexate, sulfasalazine, ciclosporin A, azathioprine, leflunomide, hydroxychloroquine and gold salts), as agents that have specific antirheumatic activities. [27]

Contrary to NSAIDs and GCs, csDMARDs are described to have a slow onset of action and are able to alter the course of RA progression, reducing or preventing joint destruction. DMARDs have also been referred to as “slowly acting antirheumatic drugs”, since the suppression of symptoms of inflammation are not apparent until months after the initiation of therapy. [5, 22, 28]

DMARDs do not have direct effects on pain relief and anti-inflammatory effects, thus they are frequently associated with NSAIDs or GCs in an early stage of RA. The exact mechanism of action of csDMARDs is still unclear, evidences indicate that these drugs modulate inflammatory and immune response. [22]

Methotrexate (MTX) is the most used csDMARD and considered the first-line anti-rheumatic agent for the past 20 years to treat both early and established RA. Opposing to most csDMARDs, MTX has a rapid onset of action, high efficacy, as well as the ease of administration and relatively low cost. [22] The mechanism of action of MTX is still not clear, but it acts primarily as an anti-folate agent, preventing lymphocytes and other inflammatory-cell mediator's proliferation, but also at the metabolic level by inhibition of purine synthesis and reduction of glutathione intracellular levels.

Recently extracellular adenosine release has been appointed as a major mechanism of anti-rheumatic effects of MTX. [29] Despite its long history of demonstrated effectiveness, reliability, sustained long-term action, high tolerability and low cost, its drug side-effect profile, often results in the cessation of therapy. [3, 30, 31] In fact, MTX adverse effects include hepatitis, cirrhosis, oral ulcers, cytopenias and interstitial pneumonitis, with particular associated renal dysfunction, since 80% of the MTX dose is excreted through the kidneys. [5]

In 2013 the European League Against Rheumatism (EULAR) recommendations for the treatment of RA support the efficacy of csDMARDs as monotherapy or in combination therapy as the initial RA treatment strategy and, preferentially in combination with GCs, at a low dose and only for a short time. [32]

Chapter 2

Nanotherapeutic Treatment

New therapeutic strategies for the RA treatment have been developed to overcome the drawbacks of drugs used in conventional therapy. RA's drugs can be highly effective in the inhibition of the inflammatory manifestation of RA. However there are still important issues that should be regarded about their efficacy and safety, especially upon long-term administration. Namely, the unfavorable pharmacokinetic behavior of these drugs, which is characterized by low bioavailability, rapid clearance rates, due to the liver metabolism, and limited selectivity either by the inability to deliver therapeutic drug efficacy to the target tissues or by potential adverse effects on normal tissues. Therefore, high and frequent dosing is often necessary to achieve an effective therapeutic in the RA inflammation tissue. Likewise, the widespread biodistribution into non-targeted tissues increases the risk of well-known systemic side effects. [1, 2, 5, 20, 33-35]

Face to the possible occurrence of these drawbacks, nanotherapy presents a selective drug delivery system approach, based on carrier system designed to specifically deliver the therapeutic active agent into the site of action (i.e., the inflammation tissue and cells). The nanocarrier selectivity may be achieved through a process known as targeting, which basically depends on spatial and temporal properties of the nanocarrier, allowing the delivery of the right amount of drug to the right place. Thereby, this may allow the reduction of necessary dosage, increasing the effective bioavailability of drug in the inflammation tissue and increasing the therapeutic efficiency. Likewise, avoiding potential systemic and non-target tissue unwanted effects. [1, 5, 35]

Furthermore, the matrix composition of the drug delivery system must be biocompatible with the drug and biological target-cell membrane barrier. [36] So, that

nanosystem has the ability to encapsulate the drug, which increases its solubility, as well as, the capability to protect biologically active drug molecules from physiological medium degradation. Thus increasing drug's stability upon blood long circulation time until reaches the target-tissue, enhancing target-cell internalization and controlling drug's release. [1, 37] Another attractive property of drug carriers is their nanometric size, which increase the surface area relatively to the volume, allowing a higher biological interaction and further promote cell internalization. [36]

Therefore, unlike conventional RA therapy, the nanotherapeutic strategy promises to increase drug stability, specificity and bioavailability and subsequently the therapeutic efficiency while reducing unwanted toxic and adverse systemic effects.

2.1 Liposomes as drug delivery strategy for RA treatment

Among the different nanotherapeutic approaches liposomes have been received a lot of interest as an advanced and versatile drug delivery systems for RA treatment.

Liposomes are self-assembled spherical vesicles composed of phospholipids, sphingolipids and/or cholesterol, derived from self-assembled enclosed on one or more lipid membrane bilayer with an internal aqueous core (Figure 3). [10, 33, 35] Once liposomes possess both lipid and aqueous phases, these versatile vesicles have the ability to carry both hydrophobic and hydrophilic drugs, respectively within the bilayer and the aqueous core. Also, due to their inherent resemblance to cell membrane-like lipid bilayer, liposomes are biodegradable and biocompatible, causing very little or no antigenic, allergic and toxic reactions. [37, 38]

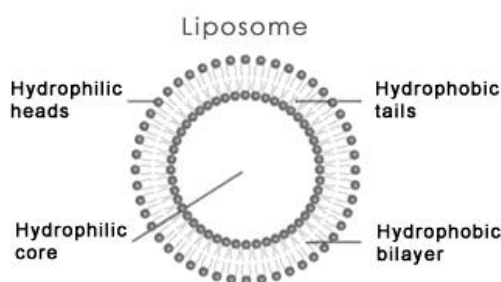


Figure 3 Schematic illustration of conventional liposomes. [39]

Liposome formation is a spontaneous process, whereas phospholipids self-assemble into lipid vesicles in an aqueous medium after stirring. [40] Phospholipids as amphiphilic molecules possess head groups which are hydrophilic and organize

themselves in such a way to point toward the aqueous core, whereas the hydrocarbon tails (hydrophobic) repelled by the water molecules are forced to face each other in the bilayer, leading to the formation of the liposome. [10, 40]

Once liposomes possess both lipid and aqueous phases, they can incorporate both hydrophobic and hydrophilic drugs within the bilayer and the aqueous core, respectively. [5, 35, 37] Therefore, liposomes simultaneously protect the host from any undesirable effects of the encapsulated drug and the therapeutic active molecules from the inactivating action of the physiological medium, preventing premature degradation of the drug by the physiological medium and, consequently, toxicity of non-target tissues. [5, 38, 41]

Furthermore, phospholipids are able to organize in a lamellar phase (i.e. bilayer structure), depending on the temperature, molecular shape of the lipids, and the conditions in the lipid-water mixture (concentration and ionic strength). Lamellar phases are classified in crystalline lamellar (L_C), lamellar gel (L_β), and lamellar liquid-crystalline (L_α). These lipid phase-transitions occur at certain temperatures according to the conditions of the medium and the type of phospholipid. [40] The phase transition (Figure 4) occurs at the temperature known as the main phase transition temperature (T_m), in which the lipid membrane passes from a tightly ordered gel (L_β) to a fluid lamellar (L_α), where the freedom of movement of individual molecules is high. [40]

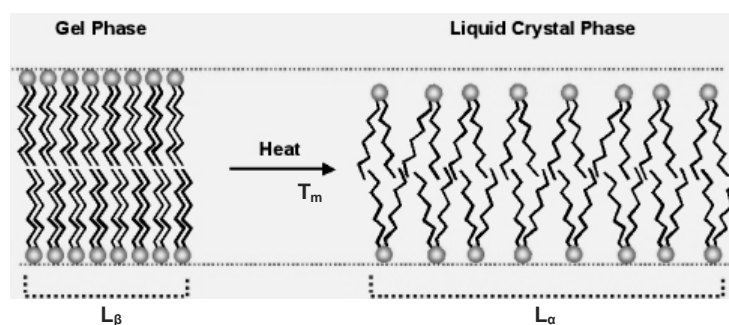


Figure 4 Representation of the phase transition of phosphatidylcholine.

Morphologically liposomes can be classified in terms of size (small or large) and number of membrane bilayers (uni or multilamellar). [10, 40] In Figure 5 are represented small unilamellar vesicles (SUV) that consist of a single lipid bilayer with an average diameter ranging from 25 to 100 nm; large unilamellar vesicles (LUV) that are also constituted by one lipid bilayer and are greater than 100 nm and multilamellar vesicles (MLV) that are made up of several concentric lipid bilayers and measure more than 500 nm. [40]

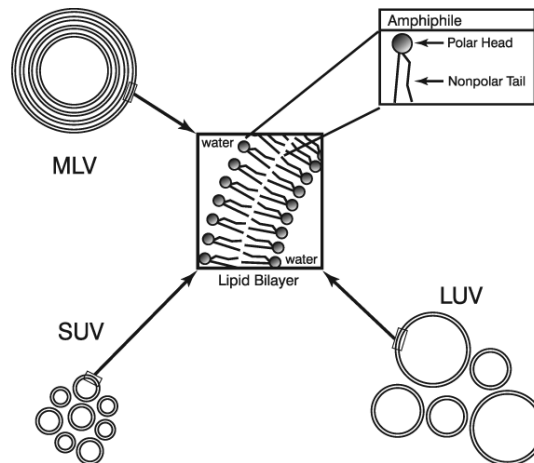


Figure 5 Classification of liposomes according to average diameter and number of bilayers. [42]

The versatile physicochemical properties of liposomes in terms of size and lipid composition can be optimized to enhance their retention at the target tissue of inflammation and hence to mediate intracellular delivery of drug molecules. Different types of lipids have varying functions, thus the type of liposome produced depends on the choice of lipid. Moreover by functionalizing the liposomes' surface bilayer with site specific ligands can also alter the biophysical characteristics of liposomes. So, it is possible to assign them targeting capability and enhance their penetration through biological target-cell membrane barrier. [5, 31, 37, 41]

As so, liposomes can be classified (Figure 6) as conventional liposomes, stealth liposomes, targeted liposomes and stimuli-sensitive liposomes.

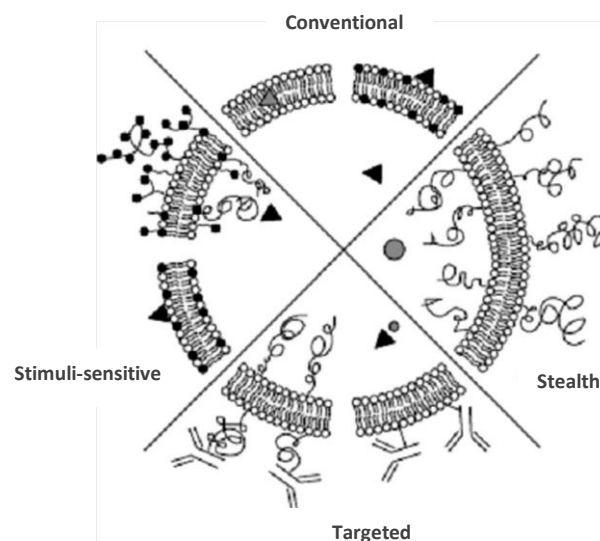


Figure 6 Representation of the four major types of liposomes. Adapted from [31].

2.2.1 Conventional liposomes

Conventional liposomes (Figure 7) are commonly composed of neutral and/or negatively charged lipids, like phosphatidylcholines, and cholesterol. [31, 38]

The major drawback of conventional liposomes is the short blood circulation time, upon systemic administration, due to the quick coating of liposomes with plasma proteins (opsonins), hence enhancing their efficient clearance by cells of the reticuloendothelial system (RES), mainly those from the liver and spleen. [5, 43] By the modification of physicochemical properties of liposomes, namely reducing the vesicle size and modulating lipid composition, to reduce uptake by RES cells, conventional liposomes can passively target inflamed tissue as a result of leaky vasculature and inadequate lymphatic drainage, an effect known as "enhanced permeation and retention" (EPR). [34, 35, 38] Although the inflamed synovial tissue, as found with RA, does not display abnormal lymphatic drainage, it's characterized by new vessel formation - angiogenesis - that enhance vascular permeability. [20, 35] Thus, the enhanced vasculature can be exploited by using EPR effect, to passively accumulate conventional liposomes within the inflamed synovial tissue. [35]

2.2.2 Stealth liposomes

In order to avoid liposomes clearance by RES cells and maximize passive targeting ability, in an attempt to further improve their binding and cellular internalization, the liposomes should be sterically stable in biological fluids. [34, 37, 43] By this means, sterically stabilized long-circulating (stealth) liposomes increase blood circulation time.

Stealth liposomes (Figure 7) can be achieved through biocompatible polymers coatings, such as polyethylene glycol (PEG) molecules. The attachment of PEG on liposome surface (PEGylation) increases the hydrophilicity of the liposome, forming a protective layer over the liposome surface and delay its recognition by plasma proteins and therefore subsequent reduction of RES cells clearance. [34, 37, 43] The steric stabilization of long-circulating liposomes results from the accumulation of highly hydrated surface PEG groups, enhancing repulsive interactions with plasma proteins and cellular biological components. [31, 44] Also, PEG enhanced liposomes' ability to passively extravasate at sites of RA inflammation by the EPR effect. [31] Above-mentioned features of PEGylated liposomes are shown in several studies as they increase the bioavailability of drugs and, as well as, allow the slow drug release into

inflamed tissue, hence reducing unwanted side effects and toxicity. [5, 33, 45-47] Therefore, stealth liposomes are suitable for encapsulation of drugs with different lipophilicities as well as for protect them until target the inflammation tissue passively.

Other biopolymers, such as polyacrylamide, polyvinyl alcohol, and polyvinylpyrrolidone, are often referred as “steric protectors”, due to their ability to protect the liposome from elimination by the RES cells. [31]

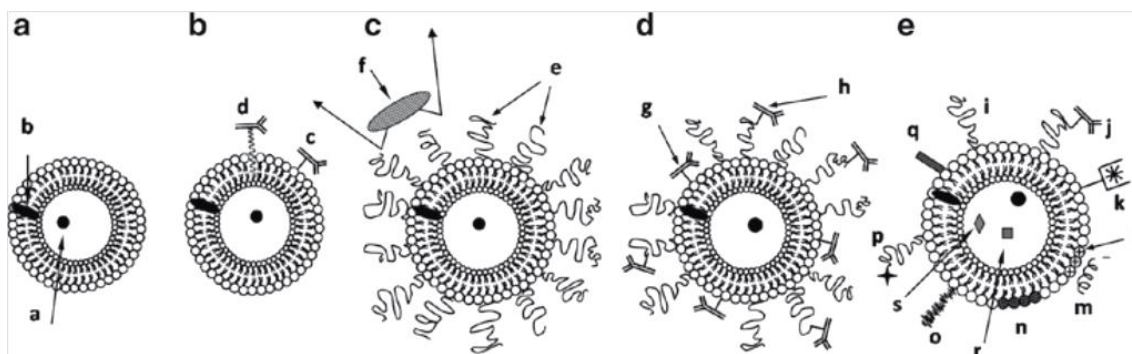


Figure 7 Schematic representation of several types of liposomes. Adapted from [38].

(a) Early conventional liposomes with water soluble drug (a) entrapped into the aqueous liposome interior, and water-insoluble drug (b) incorporated into the liposomal membrane.
 (b) Antibody-targeted immunoliposome with antibody covalently coupled (c) to the reactive phospholipids in the membrane, or hydrophobically anchored (d) into the liposomal membrane after preliminary modification with a hydrophobic moiety.
 (c) Long-circulating liposome grafted with a protective polymer (e) such as PEG, which shields the liposome surface from the interaction with opsonizing proteins (f).
 (d) Long-circulating immunoliposome simultaneously bearing both protective polymer and antibody, which can be attached to the liposome surface (g), or to the distal end of the grafted polymeric chain (h).
 (e) New-generation liposome, the surface of which can be modified (separately or simultaneously) by different ways. Among these modifications are: the attachment of protective polymer (i) or protective polymer and targeting ligand, such as antibody (j); the attachment/incorporation of the diagnostic label (k); the incorporation of positively charged lipids (l) allowing for the complexation with DNA (m); the incorporation of stimuli-sensitive lipids (n); the attachment of stimuli-sensitive polymer (o); the attachment of cell-penetrating peptide (p); the incorporation of viral components (q). In addition to a drug, liposome can be loaded with magnetic particles (r) for magnetic targeting and/or with colloidal gold or silver particles (s) for electron microscopy.

2.2.3 Targeted liposomes

The difficulty faced with liposomal delivery is the lack of specificity in targeting liposomes to sites of inflammation (i.e. organs, tissue and cells). [31] Therefore, in addition to the potential for passive targeting, active targeting can be used to increase liposomal drug accumulation in the target cells, which is essential for the therapeutic success of liposomes as liposomal drug delivery systems. [5]

To improve stability and targeting potential, surface-modified liposomes (Figure 7) are able to actively target, recognize and bind to specific cells and organs, due to the nature of their design. Thereby specific cell recognition is achieved by the high affinity

between the targeting ligands and the over-expressed receptors on target cells, therefore improving cellular internalization. [5, 31] Depending on the cell of interest to target, these ligands can be either simple molecules (such as, folic acid, transferrin) or more complex ones like antibodies (therefore, being these liposomes are also known as immunoliposomes). Moreover, the ligand can be attached by covalent binding to the liposome surface, or by electrostatic and hydrophobic insertion into the liposomal membrane, or, preferably, to the distal end of the grafted PEG chain. [40]

2.2.4 Stimuli-sensitive liposomes

Lately it have been developed a strategy to improve the ability of liposomes to mediate intracellular controlled delivery and release of drug molecules, resulting in a modified form of liposomes called stimuli-sensitive liposomes. These liposomes are able to become reactive when submitted to membrane changes triggered by pH, variations of temperature, or surface charge alterations. [40]

pH-sensitive liposomes are stable at physiological pH, but can undergo rapid liposomal destabilization acquiring fusogenic properties under acidic conditions, as presented in cells endosomes. Thus, pH trigger can provide a significant improvement on the controlled drug releasing to the cells cytoplasm. [37, 40, 43, 48]

Thermo-sensitive liposomes are vesicles that present bilayer composition in which the lipid phase-transition temperature is slightly above 37°C. Whereas, the local release of drug loaded in these liposomes is triggered by hyperthermia. [40]

Cationic liposomes (Figure 7) are composed of positively charged lipids. Due to the presence of cationic lipids at the surface give them the ability to establish favorable interactions by fusing with cellular membranes. In addition, these liposomes are suitable for the delivery of genetic material (DNA, RNA and oligonucleotides), as they can neutralize negatively charged DNA forming a more compact structure, that provides protection and promotes cellular internalization. [31, 40] Therefore, these liposomes are also known as cationic lipoplexes.

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Chapter 3

Design of pH-sensitive Liposomes loading Prednisolone for RA treatment

3.1 Prednisolone disodium phosphate

Prednisolone disodium phosphate (PDP) is a synthetic glucocorticoid, a derivative of cortisol, well-known by its anti-inflammatory and immunosuppressive effects. Thus, PDP has been widely used for suppressing pain and inflammation in RA treatment. [5, 49] This biologically active drug molecule is hydrophilic. The empirical formula is $C_{21}H_{27}Na_2O_8P$ and the molecular weight is 484.39 g/mol. [49, 50]

The oral administration route is the most often used for PDP. Despite being a more convenient, safe and economic drug delivery route, there are however some drawbacks associated with the oral route, related to the unfavorable pharmacodynamics and pharmacokinetics of the drug. [51]

3.1.1 Pharmacokinetics and pharmacodynamics

GCs like PDP are hampered by their highly unfavorable pharmacokinetic properties due to the rapid clearance owing to the liver metabolism and widespread biodistribution. This results in a low drug bioavailability and thus a decreased therapeutic efficiency. [5, 20, 34]

PDP is rapidly and well absorbed by gastrointestinal tract following oral administration. [50, 52] Systemically, PDP is quickly distributed into the kidneys, intestines, skin, liver and muscle. Although, before biodistribution in the systemic

blood circulation, PDP is mainly metabolized in the liver, being partially inactivated, and then further it's excreted in the urine as inactive metabolites. [20, 50, 51] Moreover, when PDP orally administrated passes through the gastrointestinal mucosa to reach the blood circulation, wherein contact of the drug molecules with the acidic pH of the stomach and some digestive enzymes that can change its physic-chemical properties. [36, 51] The circulating drug molecules bind extensively (70 to 90%) to protein albumin in the plasma, being only the unbound portion of a dose active. Therefore, the peak plasma concentration effects can be reached after 1-2 hours after oral administration. [49, 50]

RA conventional therapy by oral administration of PDP requires high and frequent dosing to maintain therapeutic levels at sites of inflammation. Especially upon long-term treatment, these administrations cause stomach and intestinal problems, which drastically increases the chances of patient noncompliance. [5, 20, 34, 51]

Nevertheless, GCs such as PDP, have long been used as the most potent anti-inflammatory drugs for treating inflammatory diseases, including RA. However, these drug can have both beneficial and adverse effects. In particular, PDP controls inflammatory response in RA by inhibiting the production of inflammatory cytokines and chemokines, thereby suppressing the progression of rheumatic conditions. [6] Yet, the extensive biodistribution of PDP into non-targeted tissues leads to other severe systemic adverse effects. [2, 20]

3.2 pH-sensitive liposomes

As the matrix composition is regarded, liposomes can be classified as pH-sensitive liposomes. [40] pH-sensitive liposomes are stable at physiological pH, but can undergo rapid liposomal destabilization acquiring fusogenic properties under acidic conditions, as presented in endosomes. Thus, pH trigger can provide a significant improvement on the controlled drug release to the cells cytoplasm. [37, 40, 43, 48]

The mechanism of pH trigger is relished by the physic-chemical properties of pH-sensitive liposomes, with particular emphasis in the lipid matrix composition, which influence their stability depending on the pH of the biological environment. Different classes of pH-sensitive liposomes have been proposed in the literature according to the mechanism that trigger pH-sensitivity. The most used concept involves the combination of phosphatidylethanolamine (PE), such as 1,2-dipalmitoyl-sn-glycero-3-

phosphoethanolamine (DPPE), with compounds containing an acidic group (e.g. carboxylic group) that act as a stabilizer at neutral pH. [43] In contrast to the majority of phospholipids, DPPE presents a minimally hydrated and small head group that occupies a lower volume compared to its hydrocarbon chains, exhibiting therefore cone shape geometry. [40, 43, 48] At physiologic pH (7.4) this geometry favours the formation of strong intermolecular interactions between the terminal amino group, which is positively charged, and deprotonated phosphate group (thus negatively charged) of neighboring polar head groups. Due this reason, DPPE molecules acquire an inverted hexagonal phase (H_{II}), in which the polar head group of the phospholipid points toward the inner core, while the carbon chains point toward the outer areas (Figure 8). [10, 40, 41, 48]

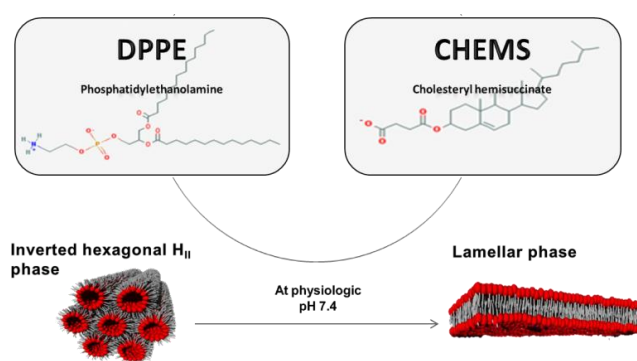


Figure 8 Main constituents of pH-sensitive liposomes, their structural representation and formation of lipid phases.

Intercalation of amphiphilic molecules such as cholesteryl hemisuccinate (CHEMS) between DPPE molecules promotes liposomal stability at physiological pH. Since the pK value of CHEMS is 5.8, its carboxylic acid would be ionized above a pH, such as pH 7.4, so that the head group is large enough to stabilize DPPE bilayers. By promoting electrostatic repulsion of the DPPE phosphate groups with the CHEMS negatively charged carboxylate groups, hence favoring the formation of the lamellar phase, which leads to liposome formation (Figure 8). [40, 43] However, when the pH is below the pK value, such as pH 5.0, the carboxylic acid would be deionized and thus the size of head group of CHEMS decrease. [53] Thereby, pH-sensitive liposomes can be design to be stable at physiological pH (pH 7.4) and undergo rapid liposomal destabilization (degradation) under mildly acidic conditions (pH 5.0), releasing the loaded drug molecules. [37, 43, 48] This mechanism of drug release (Figure 9) occurs by the exposure of liposomes to acidic pH, which leads to the protonation of carboxylate groups, then neutralizing CHEMS. Whereas, in turn DPPE molecules tend to revert

back to their inverted hexagonal phase, which hence leads to destabilization of liposomes bilayer and leakage of the encapsulated contents. [40, 43, 48]

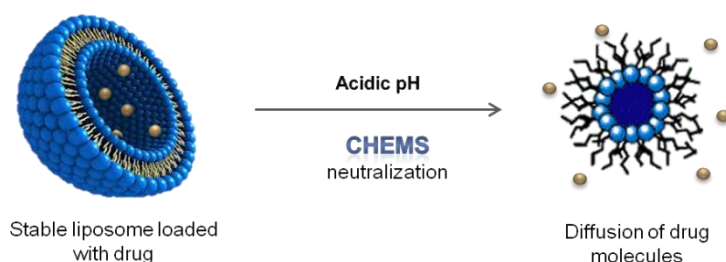


Figure 9 Mechanism of drug release of pH sensitive liposomes.

3.2.1 Administration route for pH-sensitive liposomes

Intravenous administration is the proposed route for delivery of the designed formulation of pH-sensitive liposomes encapsulating PDP, in order to diminish the cited complications of oral administration route, particularly the drug rapid clearance and the widespread biodistribution.

The main advantage of intravenous administration route over the oral one is the fact that prevents PDP of a rapid clearance avoiding its absorption by the gastrointestinal system, where the drug is unstable in the presence of gastric enzymes and acidic pH. Also, this is an additional problem to pH-sensitive liposomes which degradation depends on pH trigger. So the vascular system affords liposomal stability because bypasses processing in the gastrointestinal system and circulates directly in the bloodstream. [54]

Moreover, intravenous administration route does not allow self-medication, which route offers better control over the rate administration of design pH-sensitive liposomes and hence control over PDP pharmacokinetics. [20, 54]

3.2.2 Targeted pH-sensitive liposomes

The extent of liposome binding and internalization is a vital step in the process of intracellular drug delivery. [43] The binding and cell internalization of pH-sensitive liposomes is attributed to their tendency to form aggregates, owing to the poor hydration of DPPE head group molecules, which can explain their high affinity to adhere to cell membranes. [43] However, after intravenous administration, the major drawback of pH-sensitive liposomes is the lack of specificity and short blood

circulation time, due to rapid and efficient clearance by RES cells, mainly those in the liver and spleen. [5, 43] Therefore, the design of targeted pH-sensitive liposomes should be improved in direction to overcome this drawbacks.

The success in the design of pH-sensitive liposomes for the treatment of RA, aiming to improve the efficacy of PDP, depends on the combination of liposome pH sensitivity and cell specificity, based upon the principles of passive and active targeting. [2, 20, 35] By this means, firstly by the PEGylation of liposomes, enhancing the EPR effect and long-circulating times; secondly by modification of pH-sensitive liposomes' surface with specific targeting ligands, enabling high binding to over-expressed target cell receptors, hence ensuring cell internalization; and thirdly by using decreased endosomal pH values for trigger and control drug release. [5, 9, 20, 35]

pH-sensitive liposomes targeting strategies take advantage of the cellular roles in the synovial inflammatory environment. [55] The role of synovial cells in the clearance of systemically injected pH-sensitive liposomes, as well as, their importance in the development and progression of chronic inflammatory process, mainly by excreting a range of potent pro-inflammatory mediators and cytokines, as described in RA pathophysiology, likely make macrophages and synoviocytes found within the synovial tissue (i.e. pannus) interesting target candidates to achieve the specific therapeutic effect of targeted pH-sensitive liposomes. [35] In fact, several studies confirmed the effect of (activated) synovial macrophages and fibroblast depletion on synovial inflammation using liposomes, which induced cellular apoptosis when endocytosed and, hence, joint inflammation in arthritic animal models was effectively suppressed. [56-58]

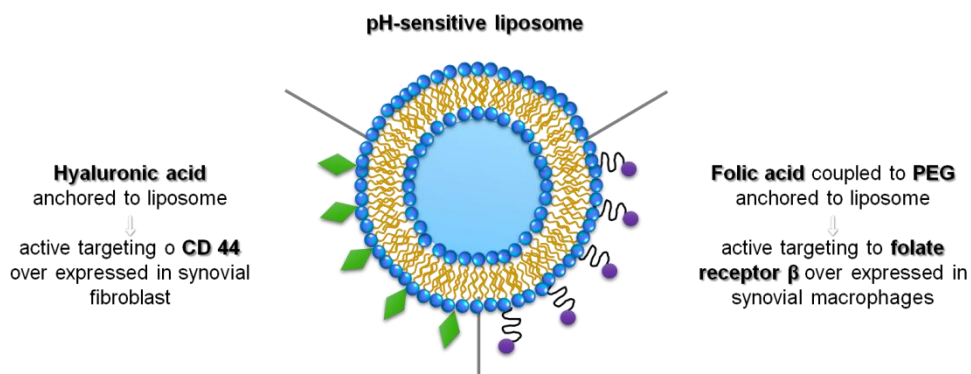


Figure 10 Designed targeted pH-sensitive liposomes.

The understanding of RA pathophysiology have led to the identification of surface receptors highly expressed by synovial macrophages and fibroblast. So, in this work were designed targeted pH-sensitive liposomes (Figure 10) modified with PEG-FA (folic acid) and hyaluronic acid (HA), since these targeting ligands selectively bind with folate and CD 44 antigen surface receptors over-expressed, respectively, in synovial macrophages and fibroblasts. Hence, this design of targeted pH-sensitive liposomes (Figure 10) enhance the potential of pH-sensitive liposomes by improving specific cellular internalization and therapeutic efficiency of PDP.

In fact, the potential on the design of pH-sensitive liposomes for the treatment of RA presented in this work (Figure 10) was overviewed to be unique from all studies reported using multiple types of designed nano systems (liposomes, polymeric micelles, dendrimers, quantum dots, polymeric, lipid and metallic nanoparticles), either involving passive or active drug targeting approaches, carried out *in vitro* as well as *in vivo* models of arthritis in various animals, for the delivery of non-biologic therapies. From this extensive research and, with the best of my knowledge, it was written and already submitted a review manuscript entitled “Non-biologic nanodelivery therapies for rheumatoid arthritis”, to be considered for publication in the journal of Biomedical Nanotechnology.

Folic Acid

Folic acid (FA) is a water-soluble vitamin that can induce receptor-mediated endocytosis. Folate receptors (FR) comprise a family of glycosyl phosphatidylinositol-anchored, high-affinity receptors for FA and there are at least 3 different isoforms: FR $_{\alpha}$, FR $_{\beta}$ and FR $_{\gamma}$. Although much research has focused on folate receptor FR $_{\alpha}$ as a target for therapy and imaging in oncology, several recent studies have used FR $_{\beta}$ as a therapeutic target on macrophages in inflammatory diseases. [59, 60] Recent studies in RA treatment have shown that the FR $_{\beta}$, which displays a high affinity for FA, is specifically expressed by activated (but not resident) synovial macrophages of various animal models of arthritis. Likewise, *in vitro* studies reported that human macrophages, taken from patients diagnosed with RA, also possess a functionally active FR $_{\beta}$. [60-66] The feasibility of using FR $_{\beta}$ to mediate the specific delivery of drug is based on several unique biologic characteristics of this receptor. Namely, the expression of FR $_{\beta}$ is specific to the macrophage lineages of hematopoietic cells, while there is negligible expression of this molecule on other blood cells, such as lymphocytes, granulocytes or

erythrocytes. [62, 67] Moreover, elevated levels of functional glycosylated FR_{β} are expressed only on activated macrophages involved in inflammatory responses, but not on quiescent resident macrophages. [60, 68] Therefore, activated synovial macrophages can be selectively targeted with FA ligands in arthritic joints, providing the possibility to specifically internalize pH-sensitive liposomes and deliver loaded PDP, without affecting normal cells and tissues. Furthermore, FA ligands can be couple to PEG on pH-sensitive liposomes, in order to unite longevity and targetability for a more stable and effective drug delivery to synovial macrophages. The PEG-linker undergoes pH-dependent cleavage in the endocytotic pathway after internalization. [35, 43]

Hyaluronic Acid

CD44 is a multistructural cell-surface glycoprotein able to generate close to 800 isoforms by differential alternative splicing. The polymorphic nature of CD44 might influence its multifunctionality and its ability to interact with many cell-surface and extracellular ligands, being the principal one hyaluronic acid (HA). [69] HA is a biodegradable, biocompatible, non-toxic, non-immunogenic and non-inflammatory linear polysaccharide, made of repeating disaccharide units of d-glucuronic acid and N-acetyl glucosamine linked by $\beta(1,4)$ and $\beta(1,3)$ glucosidic bonds. In physiological conditions, HA is in the form of a sodium salt, therefore negatively charged and referred to as sodium hyaluronate or hyaluronan. In these conditions, it is highly hydrophilic, surrounded by a sphere of water molecules linked by hydrogen bonds. [70] The biological roles of hyaluronan include the maintenance of water and protein homeostasis, and the protection of cells from the potentially harmful effects of microorganisms and macromolecules. [71] Therefore, as an alternative to replace the roles of PEG, HA has been investigated as a novel drug carrier for various protein and peptide drugs. In contrast to PEGylation, HA can be conjugated with various numbers of peptide molecules per single HA chain making possible multiple action of peptide drugs. Chemical modification and bioconjugation of HA have been carried out mostly in aqueous solution through the carboxyl groups of HA. [72]

In its native form, HA is present as a high molecular mass polymer, but during inflammation smaller molecular fragments accumulate. Fragmented HA (less than 500 kDa), rather than the high molecular mass HA (more than 1MDa) stimulates the cell-surface CD44 receptor. CD44 is critical to RA pathogenesis in leading to intracellular signaling of lymphocytes, expression of pro-inflammatory mediators and chemokines,

hence cell proliferation. [69] Low molecular fragments of HA also stimulate angiogenesis, an important factor in inflammation, which enhance liposomal accumulation in the pannus through the EPR effect. [69] Several studies have shown that the joint synovium of patients with RA contains considerable amounts of various CD44 isoforms in both synovial macrophages and fibroblasts. [69] HA has the potential to selectively bind to CD44, as it's over-expressed on the activated synovial macrophages and is involved in phagocytosis within the inflamed arthritic joint. [73, 74] The enhance expression of cell surface adhesion molecule CD44 was also found on fibroblast-like synoviocytes in the synovial pannus tissue relatively to healthy normal tissue. [73, 75, 76] Nevertheless, synovial fibroblast showed a higher expression of numerous CD44 alternatively spliced variants, including long isoforms CD44_{v3} and CD44_{v6}, which are associated with an enhanced cell internalization capacity. [77, 78] Therefore, evidence of over and/or selective expression makes the CD44 cell surface receptor suitable for HA targeted pH-sensitive liposomes in the treatment of RA.

Chapter 4

Preparation, Characterization and Study of the Designed pH-sensitive Liposomes

4.1 Preparation of designed pH-sensitive liposomes

The next chapters towards pH-sensitive liposomes preparation until the final formulations are described below. In the end, it will be obtain three liposomal formulations of DPPE:CHEMS (abbreviated to lipo), DPPE:CHEMS:PEG₂₀₀₀-FA (abbreviated to lipoFA) and DPPE:CHEMS:HA (abbreviated to lipoHA), either loading PDP or placebo. The physicochemical properties of liposomes can vary depending on the nature of lipid matrix composition. However, the same preparation method can be used to produce all designed pH-sensitive liposomes regardless of composition.

4.1.1 Thin film-hydration method

pH-sensitive liposomes loaded or unloaded with PDP were prepared by the thin-film hydration method (Figure 11), which the main steps involve lipid dissolution and hydration with agitation, followed by extrusion.

Initially, lipid solutions of 10 mM (total lipid concentration) constituted by DPPE:CHEMS (Avanti Polar Lipids, USA), DPPE:CHEMS:PEG₂₀₀₀-FA and DPPE:CHEMS:HA, respectively in a molar ratio of 6.5:3.5, 6.5:3.5:0.08 and 6.5:3.5:0.03, were prepared by dissolving the amounts of lipids in chloroform:methanol (Fisher Scientific, UK; Atom Scientific, UK) (3:2) into a round-bottom flask. [33] If it is the case,

the amount PDP power (Tokyo Chemical Industry, Japan) was dissolved in 2 mL of methanol, which was slowly added to the round-bottom flask containing the lipid mixture. [79] Then, the flask was connected to a rotary evaporation under reduced pressure of nitrogen and immersed in a warm bath with temperature maintained at 40 °C. During approximately 40 minutes, the flask was rotated and flushed with nitrogen gas to remove the solvents, thereby obtaining the thin-lipid film on the wall of the flask. For further use the films were stored in the freezer at -20°C. [33, 79, 80]

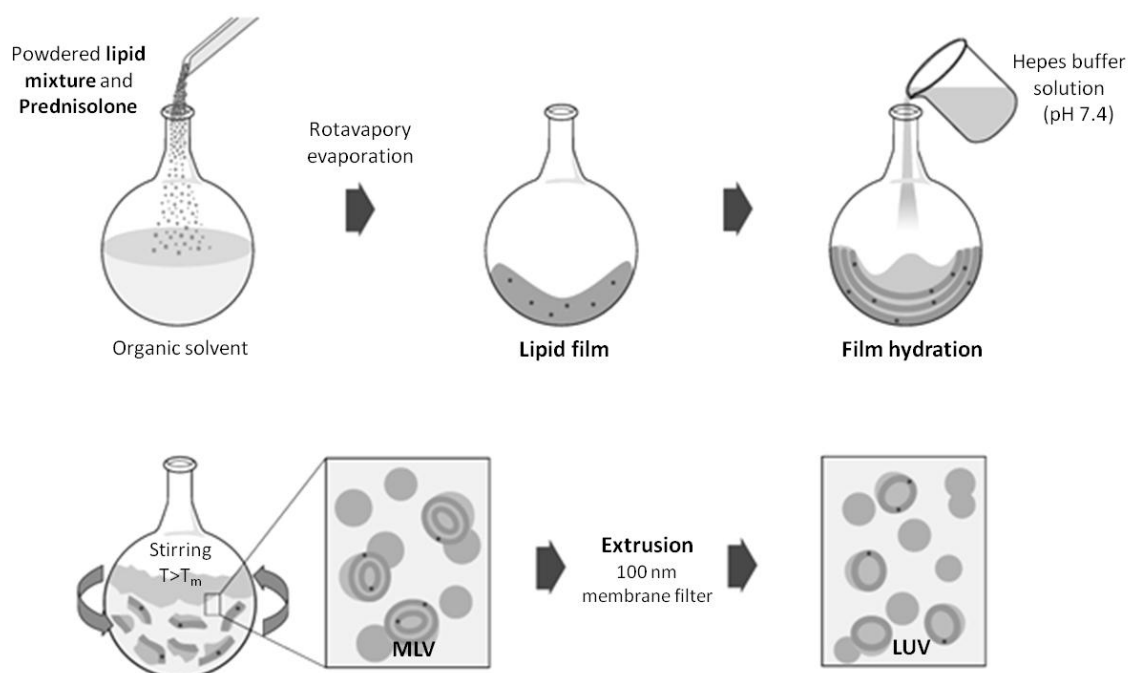


Figure 11 Representation of liposome production by thin-lipid hydration method. Adapted from [42].

The resulting dry lipid film was hydrated by the addition of adequate volume of Hepes buffered solution (10 mM Hepes, 1 M NaCl, pH 7.4). Following, it was vigorously stirred with a vortex (mechanic agitation) for 20 minutes for the spontaneous formation of MLV. In order to reach a homogeneous vesicles size distribution, the produced liposomal formulation was submitted repeatedly to extrusion. Therefore, in this technique the hydrated vesicles (5 mL Hepes buffer) are forced through a polycarbonate filter membrane (Nuclepore® Track-Etched Membranes, Whatman, UK) under pressure of nitrogen gas, with pore diameter of 600 nm three times and then 10 times through 100 nm filter at a temperature above the T_m of the lipid mixture (by this means, 65°C). [10, 33, 41, 81] A formulation of LUV of proximally 100 nm with encapsulated PDP (or not) was produced.

4.1.2 Synthesis of FA-PEG2000-DSPE conjugate

The synthesis of FA-PEG₂₀₀₀-DSPE (folic acid-poly(ethylene glycol)-distearoylphosphatidylethanolamine) conjugate can be divided in 3 main steps: activation of the folic acid, coupling to DSPE-PEG₂₀₀₀-NH₂ and purification.

Activation of folic acid

N-Hydroxysuccinimide ester of folic acid (NHS-FA) was prepared by a modified method previously described in literature, as shown in Figure 12. Briefly, 1.0 g folic acid (FA, Sigma-Aldrich, Japan) was added into a mixture of 40 mL anhydrous dimethyl sulfoxide (DMSO, Sigma-Aldrich, France) and 0.5 mL triethylamine (TEA, Sigma-Aldrich, Belgium), and FA was allowed to dissolve in the stirring mixture under anhydrous conditions and in the dark, overnight. Then the previous FA solution was mixed with 0.5 g of dicyclohexylcarbodiimide (DCC, Fluka Analytical, Sigma-Aldrich, Japan) and 0.52 g of N-hydroxysuccinimide (NHS, Sigma-Aldrich, China), and stirred in the dark for further 18 h. The side product dicyclohexylurea (DCU) precipitated was removed by filtration through a 0.45 μ m filter (Sartorius setedim biotech, Germany). DMSO and TEA were evaporated under vacuum. [82, 83]

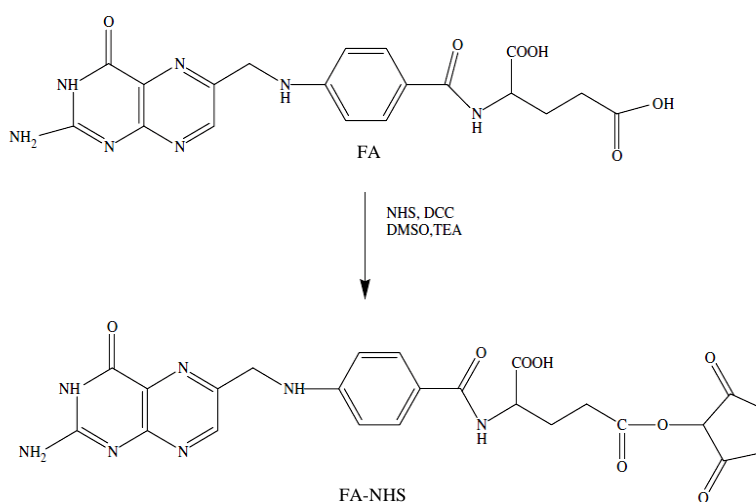


Figure 12 Scheme of the folic acid activation. Adapted from [82].

Coupling to DSPE-PEG₂₀₀₀-NH₂

FA-NHS was coupled to DSPE-PEG₂₀₀₀-NH₂ (amino-poly(ethylene glycol)-distearoylphosphatidylethanolamine, Avanti Polar Lipids, USA) as the follow described process: 2 mL of the resulting solution with activated folic acid was added to

50 mg of DSPE-PEG₂₀₀₀-NH₂ previously dissolved in 1 mL of DMSO. After the mixture was stirred in the dark overnight under anhydrous conditions, the DMSO was removed by evaporation under vacuum and 6 mL of water were added. [83]

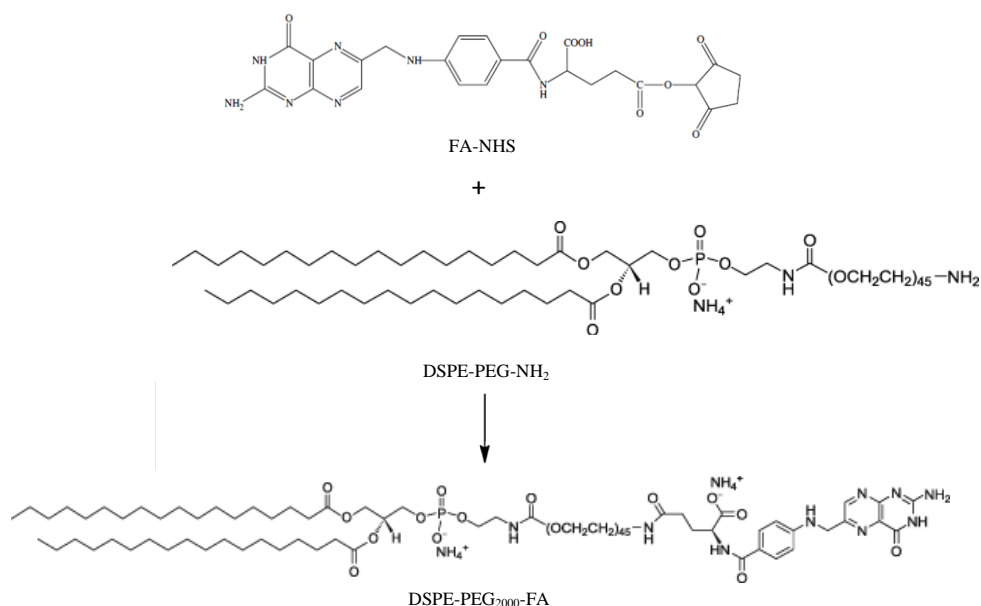


Figure 13 Scheme of the FA-NHS coupling with DPPE.

Purification of the conjugate

In order to remove the unconjugated FA, the solution of synthesized FA-PEG₂₀₀₀-DSPE conjugate (Figure 13) was dialyzed against DI water (500 mL) using a dialysis membrane with MW cut-off of 3,500 Da (Cellu.Sep T1, Regenerated Cellulose Membrane, USA) for 48 h. [82]

The resulting solution was then lyophilized in 1.0 mL aliquots using an Advantage 2.0 benchtop freeze dryer (SP Scientific, USA). Samples were frozen at -60°C in vacuum for 48 hours, yielding the FA-PEG₂₀₀₀-DSPE conjugate as a yellow dry powder, which was stored at -18°C until further use.

4.1.3 Synthesis of HA-DPPE conjugate

The synthesis of HA-DPPE conjugate can be divided in 3 main steps: activation of the hyaluronic acid, coupling to DPPE and purification.

Activation of hyaluronic acid

HA with amine-reactive functional groups can be prepared by the conjugation of HA-COOH with NHS. According with previously reported methods in literature, the

process began with the activation of the hydroxysuccinimide ester of hyaluronic acid (NHS-HA). Briefly, 1.0 mg of HA (salt form, 300-420 kDa, a kind gift Genzyme Corporation, UK) was dissolved in 50 mL of water, followed by incubation with 0.5 g of ethyldimethyl-aminopropyl-carbodiimide (EDC, Sigma-Aldrich, Japan) and 0.52 g of NHS at pH 4 (adjusted with 1 M HCl) for 2 hours at 37°C.

Coupling to DPPE

At the end of the incubation, 100 mg of DPPE (Avanti Polar Lipids, USA) were added to the resulting solution with activated HA, buffered by 0.1 M borate buffer at pH 8.6. The reaction was maintained for 24 hours at 37°C. [84-89]

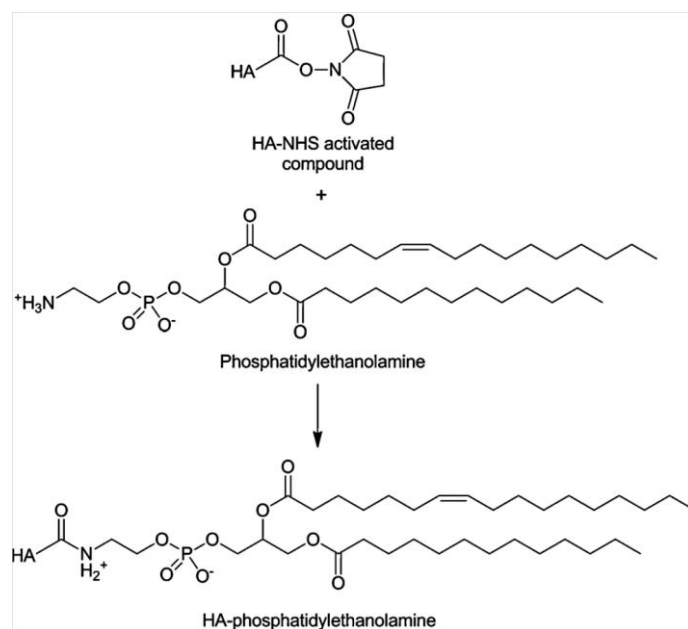


Figure 14 Scheme of the HA-NHS coupling with DPPE. Adapted from [70].

Purification of the conjugate

The solution of HA-DPPE conjugate (Figure 14) was purified to remove the excess of unconjugated HA and by-products by centrifugation (5500 rpm, 4°C and 40 minutes) and repeatedly wash with PBS (Dulbecco's phosphate buffered saline, Sigma-Aldrich, USA), reducing the pH back to 7.4. [84-87]

The resulting solution was then lyophilized in 1.0 mL aliquots using an Advantage 2.0 benchtop freeze dryer. Samples were frozen at -60°C in vacuum for 48 hours, yielding the HA-DPPE conjugate as a white dry powder, which was stored at -18°C until further use.

4.2 Physicochemical characterization of designed pH-sensitive liposomes

Both placebo and PDP loaded non-modified and modified pH-sensitive liposomes, either with PEG₂₀₀₀-FA or HA were characterized chemically in terms of encapsulation efficiency, drug loading capacity and physically by the vesicles size, size distribution, zeta potential and morphology.

4.2.1 Encapsulation Efficiency and Drug Loading Capacity

Encapsulation efficiency (*EE*) and loading capacity (*LC*) is an essential parameter to evaluate liposomes as drug delivery systems. The *EE* is the ratio between the amount of drug encapsulated within the liposomes and the total amount of drug added to the liposomal solution at the beginning of the preparation method. On the other hand, the *LC* is the ratio between the amount of drug loaded in the liposomes and the total amount of lipids added to the liposomal formulation. [40]

$$EE (\%) = \frac{\text{Amount of drug in the liposome}}{\text{Total amount of drug}} \quad (1)$$

$$LC (\%) = \frac{\text{Amount of drug in the liposome}}{\text{Total amount of lipid}} \quad (2)$$

In order to determine the amount of entrapped drug, the liposomal formulations (aliquots with 1.5 mL of each one), in Amicon ultra centrifugal tubes with filters of 100 nm (Merck Millipore, Germany), were centrifuged at 3500 rpm for 10 minutes at 25°C. Prior to the centrifugation, the samples were diluted (1:50) using Hepes buffered solution. For each liposomal formulation, the result supernatant was removed and the amount of free drug analyzed using a UV/Vis spectrophotometer (Jasco V-660, Japan). This is one of the most useful techniques available for quantitative analysis, thereby being important for determining the concentration/amount of free drug (non-encapsulated in the liposomes). [90] In the end, the amount of encapsulated drug corresponds to the subtraction of total amount of PDP in the formulation to of free drug analyzed in the supernatant.

In order to establish the relationship between absorbance and concentration a calibration method was needed. The calibration curve was obtained from a series of standard solutions that encompass the concentration range expected for the sample

solution. The standard solutions for calibration approximated the overall composition of the samples (analyte and matrix concentrations) as closely as possible, minimizing interference effects of components' sample on the measured absorbance. [90] From the absorption spectrum of the standard solutions of PDP diluted in Hepes or Acetate buffer was selected a wavelength that correspond to the prominent absorbance peak. Then, in Figure 15 is presented the linear fit- calibration curve - showing a high R^2 value that was obtained by plotting absorbance measured against concentration of the standard solutions. Likewise, the amount of PDP encapsulated in the developed liposomal samples was quantified by measuring the absorbance at 247 nm (corresponding to the peak in the absorption spectrum for highest absorbance of PDP concentration) of the supernatant solutions. Before each sample measurement, a baseline with Hepes or Acetate buffered solution was performed.

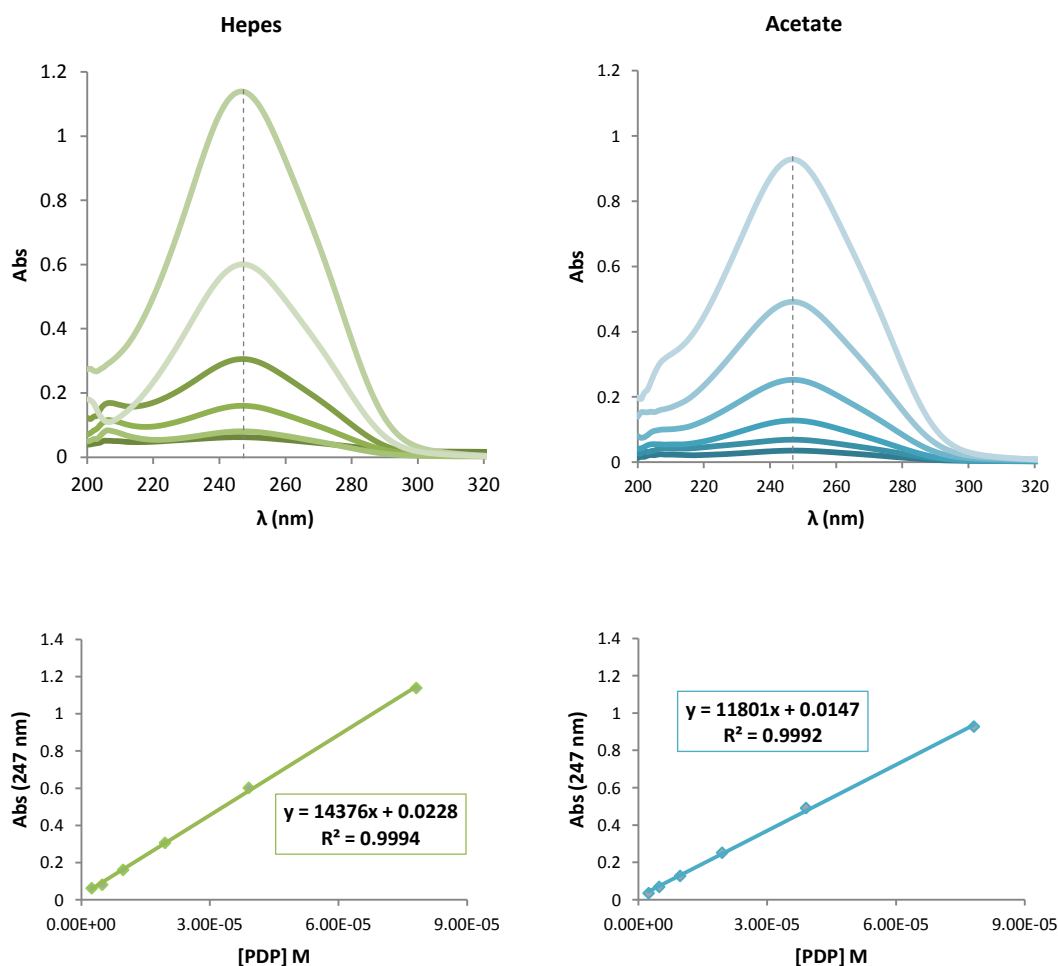


Figure 15 Spectrum of PDP standard solutions in Hepes (left) and Acetate (right), and the respective calibration curves resultant of the plot of their concentration versus absorbance at 247 nm.

For each developed liposomal formulations (DPPE:CHEMS, DPPE:CHEMS:PEG₂₀₀₀-FA and DPPE:CHEMS:HA) loading PDP at least 3 independent sample measurements were performed and both % EE and %LC were determined, respectively with equations (1) and (2), by indicating the mean percentage and the standard deviation (SD).

4.2.2 Size and size distribution

Dynamic Light Scattering (DLS), sometimes referred to as Quasi-Elastic Light Scattering (QELS) or Photon Correlation Spectroscopy (PCS), is a non-invasive and a well-established technique to determine the hydrodynamic size and size distribution of particles, typically in the nano region, such as liposomes. Disperse liposomes suspended in a liquid medium undergo in Brownian random motion, which cause laser light to be scattered at different intensities. DLS through autocorrelation functions and software calculations measures the dynamic fluctuations of light scattering intensity as a function of time, in order to give information about the mean hydrodynamic diameter and polydispersity index of liposomes in suspension. [91]

The mean hydrodynamic diameter and polydispersity index of developed liposomal formulations (DPPE:CHEMS, DPPE:CHEMS:PEG₂₀₀₀-FA and DPPE:CHEMS:HA) loading PDP and placebo samples were determined with a Nanoparticle Size Analyzer from Brookhaven Instruments (90Plus, New York, USA). Measurements were recorded using a dynamic light scattering apparatus at 6°C and at a scattering angle of 90°. Prior to the measurements, the samples were diluted (1:60) using Hepes buffered solution in order to yield a suitable scattering intensity and dismiss sizing or distribution width measurements due to interference effects of an inadequate concentration of sample solution.

For each sample 6 measurements were performed and 3 independent formulation sample measurements were carried out to achieve statistical significance. Also, both hydrodynamic size and polydispersity index measurements of each liposomal formulation were determined by calculating the average and indicating the standard deviation (SD).

4.2.3 Zeta potential

Phase Analysis Light Scattering (PALS), also referred as Electrophoretic Light Scattering (ELS) is a well-established method, due to its sensitivity, accuracy and versatility, to determine the zeta potential of liposomes. [92]

The liquid layer surrounding the particle is formed by two parts: an inner region, called the Stern layer, where the ions are strongly bound; and an outer, more diffuse region where the ions are less firmly attached. Within this diffuse layer there is a notional boundary inside, which the ions and particles form a stable entity. When a particle moves, ions within the boundary move with it, but any ions beyond the boundary do not travel with the particle. This boundary is called the surface of hydrodynamic shear (or slipping plane) and the potential that exists at this boundary is known as the zeta potential (Figure 16). [92-94]

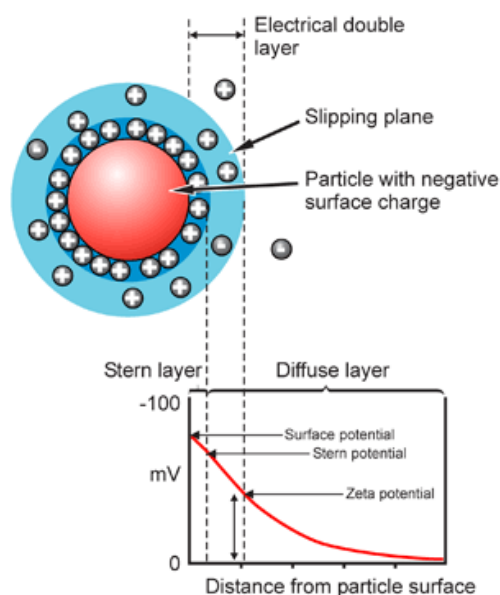


Figure 16 Schematic representation of the zeta potential of a negatively charged particle. [94]

The application of an electric field across the liposomes in suspension, using electrodes, results in a directed migration of the liposomes to the electrode of opposite charge, overlapping their Brownian motion. When, the laser passes through the cell sample, occurs electrophoresis and frequency shift of the scattered laser light, which is proportional to electrophoretic mobility of the liposomes. ELS through autocorrelation functions and software calculations measure the electrophoretic mobility of liposomes in suspension, in order to obtain the zeta potential liposomes. [92-94]

Nevertheless, zeta potential, unlike particle size, is a property that involves not only the liposomes but also their environment, namely pH, ionic strength and type of ions in solution. [92] Therefore, it's a critical parameter for the stability of the liposomal formulation and for assess liposomes charge surface and interactions, either between liposome-liposome or liposome-cell. [95]

The zeta potential of the developed liposomal formulations (DPPE:CHEMS, DPPE:CHEMS:PEG₂₀₀₀-FA and DPPE:CHEMS:HA) loading PDP and placebo were determined with a Nanoparticle Zeta Potential Analyzer from Brookhaven Instruments Corporation (ZetaPALS, Holtsville, New York, USA). Measurements were recorded at 6°C with an inert electrode using phase analysis light scattering mode at an angle of 90°. Prior to the measurements, the samples were diluted (1:60) using Hepes buffered solution.

For each sample 6 measurements were performed and 3 independent formulation sample measurements were conducted to achieve statistical significance. Also, the zeta potential measurement of each liposomal formulation was determined by calculating the average and indicating the standard deviation (SD).

4.2.4 Morphology

To evaluate the liposome's morphology and matrix structure resulting from changes in pH, all design liposomal formulations (DPPE:CHEMS, DPPE:CHEMS:PEG₂₀₀₀-FA and DPPE:CHEMS:HA) were examined using a transmission electron microscopy (TEM, Jeol JEM 1400, Tokyo, Japan). TEM is a technique that focus a high-energy beam of electrons, at the accelerating voltage of 60 kV, through a thin sample. Resulted from this interaction, the non-uniform distribution of scattered electrons contained the morphologic, compositional and crystallographic information of the sample. This information can be viewed by a specific distribution of scattering which generates a contrast image of the sample. [96] Images were digitally recorded using a Gatan SC 1000 ORIUS CCD camera (Warrendale, PA, USA), and photomontages were performed using Adobe Photoshop CS software (Adobe Systems, San Jose, CA).

The samples (10 µL of the liposomal dispersion diluted in 1:10) were mounted on copper support grids, that possess an ultramicrotomy 300 mesh with dimensions of 3 mm of diameter, 100 µm of edge thickness, and electron transparent in the mesh

region. Then, after 1 minute the excess was removed and the sample was stained with an aqueous solution of 1% uranyl acetate for 5 seconds. Uranyl acetate is usually used as a negative staining for samples of biological origin, since it deposits uranium atoms in specific regions of the specimen in order to absorb electrons from the beam, hence enhancing the image contrast.

4.3 Stability studies

The stability studies consists of weekly measurements of the %EE, %LC, hydrodynamic size, polydispersity index and zeta potential of all designed liposomal formulation (DPPE:CHEMS, DPPE:CHEMS:PEG₂₀₀₀-FA and DPPE:CHEMS:HA) put in storage at 4°C protected from light for at least one month.

The physical stability of pH-sensitive liposomes strongly depends on the vesicle size and liposome width distribution, because these parameters influence liposomes biodistribution and stability *in vivo*, as these factors can determine time circulation of liposomes in the bloodstream before liposomal disintegration until reach target cells. [40]

The analysis of zeta potential, is also an important parameter, once the nature and charge density of liposomes' surface influence the mechanism and extent liposome-cell interactions. Likewise, it is also important to assess the electrostatic interactions between liposomes due to the possible occurrence of aggregation phenomena. [40]

The instability of the liposomal formulation is thereby allied to the increase in size due to the aggregation of unstable liposomes during the formulation processing or/and upon storage. An increase in vesicle size of liposomes generally results in rapid uptake by the RES cells with subsequent rapid clearance and a short-life of liposomes. [81]

Moreover, the physical stability also influence the chemical stability of the drug loaded in the liposomes, by means that determine the amount of bioavailable drug for therapeutic efficiency, thus the measurement of the %EE and %LC are parameters that have to be taken into account upon liposomal storage over time. [40]

Therefore, controlling and maintaining liposomes at uniform sizes, constant zeta potential and suitable drug loading, in storage conditions are critical in developing a stable pharmaceutical formulation. [40]

The stability study was performed in triplicate for each liposomal formulation to achieve statistical significance and weekly measurements were carried out during at least 1 month. Also, the measurements of each stability parameter were determined by calculating the average and indicating the standard deviation (SD).

4.4 Drug release study

Although the above described set of techniques is essential, they are insufficient for a complete characterization of designed pH-sensitive liposomes. As the aim reason for pursuing nanotherapeutic strategy is to deliver drugs into target-cells, hence for an effective drug action is of much importance understanding the mechanism and extent to which the drug molecules are released from liposomes into cells cytoplasm. Thereby, *in vitro* drug release studies are an important parameter in controlling the therapeutic efficiency of designed pH-sensitive liposomes as drug delivery systems. Likewise, it can be used for the prediction of *in vivo* drug release profile.

Furthermore, being the property that triggers the mechanism of liposomes to mediate intracellular controlled release of drug active molecules the lipid's pH sensitivity to mildly acidic conditions, as the ones present in endosomes of RA target-cells in which they are internalized, it is crucial that the dissolution medium mimics the pH and salt concentrations in the endosomes (i.e. liposomes dissolved in Acetate buffered solution at pH 5.0). Furthermore, to evaluate the drug release that the liposomal formulation under physiological conditions upon intravenous administration in the bloodstream, the liposomes were suspended in Hepes buffered solution (pH 7.4). [37, 43, 48]

The *in vitro* release study of PDP was carried out by the dialysis assay, which was performed using a cellulose ester dialysis membrane (Spectra/Por, Float-A-Lyzer G2, Spectrum Laboratories, Germany) with a nominal molecular weight cut off of 3.5 - 5 kDa, filled with 0.5 mL of sample. The samples were maintained in sink conditions under 80 mL of each buffer solution with distinct pH values at 300 rpm stirring and 37°C. At regular time intervals, 1 mL aliquots were collected and replaced with the same buffer to maintain the sink conditions. The amount of PDP released was quantified using a UV/Vis spectrophotometer at 200 to 600 nm and calculated using the drug calibration curve.

The drug release study was conducted in triplicate for each liposomal formulation to achieve statistical significance and the cumulative percentage of released drug was determined by calculating the average between the measurements, indicating the standard deviation (SD).

4.5 pH-dependent calcein study

Calcein is a water-soluble compound that is stable and highly fluorescent, with a maximum absorption at 495 nm and maximum emission at 515 nm. This fluorescent dye is known to be self-quenched at a higher concentration (80 mM or higher). [37, 97] Thus, higher quenching means that the concentration of calcein is high enough to suppress its fluorescence. This quenching phenomenon occurs when a concentrated calcein solution is enclosed in vesicles, such as, liposomes. [53]

The liposomal membrane integrity and permeability was evaluated by the pH-dependent calcein assay, to detect any process that causes leakage of aqueous contents, including fusion, lysis or permeabilization. Thereby, since the intensity of fluorescence of calcein release strongly depends on the pH, upon addition of a destabilizing agent, the dye release is accompanied by an increase in intensity of fluorescence. [53]

A concentrated solution of calcein (Sigma-Aldrich, Japan) was encapsulated in non-modified pH-sensitive liposomes, which were previously prepared through extrusion (Hamilton, Avanti Polar Lipids, USA) with a 100 nm polycarbonate filter membrane (Nuclepore® Track-Etched Membranes, Whatman, UK) and, then separated from any remaining free dye by gel filtration chromatography (Sephadex G-25, Sigma-Aldrich, Sweden). Freshly column purified liposomes entrapping calcein (1.75 mM, 275-300 mOsm) and 10 mM of lipids were added to different buffer solutions with increasing pH range (pH 5.0, 1 M Acetate buffer and pH 7.4, 275-300mOsm, 10 mM Hepes/4 M NaCl) and, to 10, 40, 100 μ M of PDP solution. Whereas, complete dye release was obtained by lysing the liposomes with 1% Triton X-100 (Sigma-Aldrich, USA), which can be used to determine the assay control. The release of the fluorescent dye calcein was monitored with a Synergy™ HT Multi-mode Microplate Reader (BioTek Instruments, USA), where intensity fluorescence measurements were carried out at 37°C and with excitation and emission wavelengths set at 495 and 515 nm.

The percentage of total fluorescence of calcein release and quenching of calcein fluorescence loaded in liposomes were determined as follows:

$$\% \text{ release} = \frac{I_t - I_0}{I_f - I_0} \times 100 \quad (3)$$

$$\% \text{ quenching} = 1 - \frac{I_i}{I_f} \times 100 \quad (4)$$

Where I_0 is the initial intensity of fluorescence at pH 7.4, I_f the total fluorescence observed after addition of Triton X-100, I_i is the initial fluorescence after removing free calcein and I_t the fluorescence at a given pH. [53]

4.6 Cellular studies

The inflamed RA target cells, macrophages and fibroblast, are the main responsible cells in the inflammation process. Thereby, *in vitro* studies were carried out on both murine macrophage RAW 264.3 and fibroblast L929 cell lines to evaluate the delivery of PDP loaded on designed pH-sensitive liposomes using the MTT viability assay and LDH cytotoxicity assay. As well as, it was investigated the cellular uptake kinetic between all designed pH-sensitive liposomes in each cell line.

4.6.1 Cell culture growth

Both RAW and L929 cell lines were cultured in Dulbecco's Modified Eagle medium (DMEM, Gibco, Lifetecnologies Corporation, USA) supplemented with 10% fetal bovine serum (FBS, Lifetecnologies Corporation, USA) and 5% penicillin-streptomycin (Pen-Srep, Gibco, Lifetecnologies Corporation, USA). Cells were allowed to grow at 37°C, 5% CO₂ and 95% humidity. To allow cell confluence passages were performed. Subculturing of L929 and RAW cells involved the double washing with PBS at pH 7.4, followed by detachment using a scraper and resuspension in fresh culture DMEM medium. Subculture was done at a proportion 1:6 or 1:4 at least once a week, in order to achieve 80-90% cell confluence to performed *in vitro* assays.

4.6.2 MTT assay

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, USA) is a yellow water soluble tetrazolium salt that may be used in measurement of metabolic activity of the mitochondria. [98] Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by

active mitochondrial dehydrogenases of living cells. The amount of formazan (purple) produced is proportional to the number of living cells and it can be quantified by absorbance measurements in the range 550 to 600 nm. [99] MTT is a rapid and easy assay that has been widely used to assess cell viability. However, there are some factors that have to be taken in account when performing an MTT assay, as it may comprise cell density, culture medium, optimal concentration and exposure time for MTT among others. [100] Therefore, a range of concentrations, as well as, different exposure times of MTT should be test and well establish with controls. [99]

PDP cytotoxicity was evaluated in both RAW and L929 cell lines using the MTT viability assay, since actively living cells convert the water-soluble MTT to an insoluble purple formazan that then can be quantified, allowing to assess the metabolic activity and therefore the viability of cells when exposed to designed pH-sensitive liposomes or free drug. Briefly, cells were seeded at 5×10^4 cell per well in 96-well plates and incubated for 3 hour at 37 °C, 5% CO₂, during which cells were attached and resumed to grow. Meanwhile, the liposomal formulations were diluted with culture medium to achieve various concentration of liposomes (either loading PDP or placebo) and free PDP, with which cells were treated (150 µL in each well) and plates incubated for 24 hours at 37 °C, 5% CO₂. Control wells were treated with equivalent volumes of fresh DMEM and 2% Triton X-100 in PBS. After 24 hours, the supernatant was removed and stored at 4°C. MTT (0.5 mg/mL) in culture medium was added to each well (200 µL) and incubated for 2 hours at 37 °C, 5% CO₂. Then, after the unreduced MTT was discarded, was added to each well 200 µL of DMSO to dissolve the formazan crystals. Cellular viability was determined by measuring absorbance values at 590 and 630 nm using a Synergy™ HT Multi-mode Microplate Reader. The percentage of cell viability was calculated comparing the absorbance of each well containing the treated cells with the nontreated control cells ($Abs_{\text{DMEM control}} - Abs_{\text{Triton X-100 control}}$), according to the formula: % cell viability = (means $Abs_{\text{treated cells}}$ /means $Abs_{\text{nontreated cells}}$) \times 100 ; and indicating the standard deviation (SD). To achieve statistical significance, two independent MTT assays with five replicas each were conducted for each liposomal formulation and free PDP.

The concentrations of PDP leading to 50% cell growth inhibition (IC₅₀) were them derived from MTT assay results. A linear regression was performed and the equation that correlates the concentration with the cell viability was used to calculate IC₅₀ by interpolation.

4.6.3 LDH assay

Cell death is assayed by the quantification of plasma membrane damage. Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme which is present in most cells. It is released into the cell culture supernatant upon damage of the cytoplasmic membrane. LDH cytotoxicity detection kit (Takara Bio, Japan) allows precise, fast and simple colorimetric assay method to quantify cytotoxicity based on the measurement of LDH activity released from the of damage cells into the supernatant, by the LDH activity is based on the reduction of formazan by cleavage of the tetrazolium ring by active enzymatic diaphorase of damage cells. The amount of formazan dye (red) formed is proportional to the number of damage cells and it can be quantified by absorbance measurements, yielding the maximum of absorption at about 500 nm.

LDH assay was conducted in order to evaluate the cytotoxic effect of the design pH-sensitive liposomes (either loading PDP or placebo) and more specifically cell death levels after PDP exposition. The surrounding cell culture supernatant was collected cell-free, from the MTT assay previously performed. Plates containing this supernatant were centrifuged (250 g, 10 minutes, 20°C) to remove any wastes and cellular debris and also the liposomes. From each well, 100 µL of sample from the supernatant were taken and analyze for LDH activity according to the manufacturer's instructions of the LDH cytotoxicity detection kit. Briefly, 100 µL of the reaction mixture of the LDH kit were added to the 100 µL of sample. After 15 minutes and protected from light, the samples absorbance values were measured at 490 nm and 630 nm in a Synergy™ HT Multi-mode Microplate Reader. The percentage of cytotoxicity was calculated comparing the absorbance of each well containing the treated cells with the nontreated control cells ($Abs_{Triton\ X-100\ control} - Abs_{DMEM\ control}$), according to the formula: $\% \text{ cytotoxicity} = (\text{means } Abs_{treated\ cells} / \text{means } Abs_{nontreated\ cells}) \times 100$; and indicating the standard deviation (SD). To achieve statistical significance, two independent LDH assays with five replicas each were conducted for each liposomal formulation and free PDP.

4.6.4 Uptake kinetics assay

The behavior of the designed pH-sensitive liposomes targetability determine their mechanism interaction with cells of interest, it's thereby necessary a clear understanding of the cellular uptake kinetics of liposomes.

Liposomal formulations with fluorescent characteristics were produced by the addition of 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2,1,3-benzoxadiazol-4-yl) (NBD-DPPE, Avanti Polar Lipids, USA). NBD was added at 1 mol % of the amount of lipid and added to the organic phase when thin-lipid film preparation. Then, liposomes were produced as previously described.

The observation of uptake behavior of fluorescent pH-sensitive liposomes in target cell lines (both RAW and L929) was studied by flow cytometry using a BD Accuri C6 (BD Biosciences, Belgium). Cells were seeded in 24-well plates at the density of 10^5 cells per well and incubated for 20 h to allow for cell attachment. To study the effect of incubation time, the DMEM medium was replaced with 400 μ L medium containing 1 mM of each liposomal formulation, and the cells were incubated for 0.5, 1, 2, 4, 16, and 24 h, respectively. After each liposomes' incubation times, the cells were washed twice with PBS, followed by trypsinization to detach cells. Whereas, in each well 200 μ L of trypsin (Sigma-Aldrich, USA) were added and incubated for 5 min at 37°C. Then, to remove non-internalized NBD-liposomes, cells were recovered in 200 μ L of fresh DMEM medium containing with 0.11% Trypan Blue (Sigma-Aldrich, USA) and incubated for 1 min in order to quench the NBD-fluorescent signal coming from non-internalized liposomes.

Cells were examined under the 488 nm excitation and 530 nm emission wavelengths in the BD Accuri C6 flow cytometer. For each sample a minimum of 10,000 events were recorded and the auto-fluorescence of non-treated cells were used as control. The cellular uptake of fluorescent liposomes was expressed as the geometric mean fluorescence intensity of liposomes into target cells. Data were analyzed with BD Accuri C6 Software (BD Biosciences, Belgium).

This assay was only conducted one time with 2 replicas of each designed liposomal formulation for each incubation time.

4.7 Statistical analysis

Statistical analysis were performed using SPSS software (v22.0; IBM, Armonk, NY, USA). The measurements were repeated at least three times and data were expressed as mean \pm SD. Data were analyzed using one-way analysis of variance (oneway ANOVA), followed by Tukey post-hoc tests. A p value of < 0.05 was considered statistically significant.

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Chapter 5

Results and Discussion

5.1 Physicochemical characterization of designed pH-sensitive liposomes

All designed pH-sensitive liposomes (lipo, lipoPEG-FA and lipoHA) loading PDP and placebo were characterized chemically in terms of %EE, %LC and also physically by the vesicle size, size distribution, zeta potential and morphology. The following sections presents the results obtained. Designing the pharmaceutical liposomal formulations for RA treatment by surface modification of pH-sensitive liposomes either with PEG₂₀₀₀-FA or HA specific targeting ligands, the aforementioned parameters necessarily change, inevitably influencing liposomes biological performance. [34, 37]

5.1.1 Optimization on the development of pH-sensitive liposomes

The experimental part of this dissertation was initiated with the optimization of pH-sensitive liposomes development in terms of %EE, %LC, hydrodynamic size, polydispersity index and zeta potential.

The first step towards this process of optimization was to determine the suitable amount of PDP (12 mg, 16 mg and 20 mg) loaded in the liposomes. In this context, %EE and %LC were the first parameters to be optimized in non-modified pH-sensitive liposomes. EE is related with the amount of PDP encapsulated within designed pH-sensitive liposomes, while LC relates the amount of drug with the amount of lipid. Two different methods were performed to measure the %EE and %LC and taken in account in the optimization process. One of the methods involved the dialysis assay

using a suitable dialysis membrane with MW cut-off of 12-14 kDa (Cellu.Sep T3, Regenerated Cellulose Membrane, USA), that during 5 h allowed the free drug release, while the other method used Amicon ultra centrifugal tubes filters submitted to centrifugation to separate free PDP from the drug loaded into liposomes. Since the results of %EE and %LC were the same in both methods, the last one was found to be more suitable once it allowed a faster result. The parameters of centrifugation in terms of velocity and time were also refined in order to not promote PDP release from the liposomes which would lead to incorrect %EE and %LC.

Then, after the achievement of a suitable method to measure the %EE and %LC it was unquestionable that the pH-sensitive liposomes were capable of incorporating a higher amount of PDP (20 mg) without compromising %EE and increasing drug %LC (Table 1), which is indeed a more important feature as it relates the amount of drug loaded in the liposomes with the total amount of lipids added to the liposomal formulation. In accordance with this, the amount of PDP was established to a concentration of 4 mg/mL in a 10 mM concentration of liposomal formulation.

Table 1 Amount of PDP versus %EE and %LC of liposomes.

Amount of PDP	% EE	% LC
12 mg	74%±1%	27%±2%
16 mg	74%±2%	38%±1%
20 mg	70%±2%	45%±1%

All data express the average ± standard deviation (n=3).

5.1.2 Encapsulation efficiency, drug loading capacity, hydrodynamic size, polydispersity index and zeta potential

The physicochemical characterization of all designed pH-sensitive liposomes was evaluated in terms of %EE, %LC, hydrodynamic size, polydispersity index and zeta potential.

Encapsulation efficiency and drug loading capacity

%EE and %LC experimental results for all the developed liposomal formulations are presented in Table 2. The results show a 70% of EE and 45% of drug LC of designed pH-sensitive liposomes. Moreover, the modified pH-sensitive liposomes either with PEG₂₀₀₀-FA or HA do not change the percentage of EE and drug LC of liposomes,

which indeed it's a positive remark on the chemical characterization of the designed liposomal formulations. By this means that 45% from the initial amount of PDP added to the liposomal formulation, proximally, 9 mg would be available for therapeutic efficiency. In fact, ≤ 10 mg is the dose of GC drug indicated per day to treat 30 to 60% of patients for joint disease. [101] Taking into account the drug pharmacokinetics, only 10 to 30% of that dose (i.e. 1 to 3 mg) unbound to plasma protein albumin would be active for therapeutic efficiency. Therefore, for all designed pH-sensitive liposomes, 45% of drug available is a satisfactory high result.

Table 2 Designed pH-sensitive liposomes %EE, %LC, hydrodynamic size diameter, polydispersity index and zeta potential.

		% EE	% LC	Diameter (nm)	Polydispersity Index	Zeta potential (mV)
lipo	PDP	70 \pm 2	45 \pm 1	104 \pm 3*	0.06 \pm 0.03	- 33 \pm 2
	Placebo	-	-	110 \pm 1*	0.05 \pm 0.01	-36 \pm 1
lipoPEG-FA	PDP	70 \pm 1	45 \pm 1	105 \pm 3* [#]	0.12 \pm 0.01 [#]	- 35 \pm 2
	Placebo	-	-	111 \pm 2*	0.11 \pm 0.02 [#]	-33 \pm 3
lipoHA	PDP	70 \pm 1	45 \pm 1	116 \pm 3 [#]	0.08 \pm 0.02	- 37 \pm 3*
	Placebo	-	-	114 \pm 3	0.10 \pm 0.01 [#]	-45 \pm 2* [#]

All data express the average \pm standard deviation (n=3 for liposomes loading PDP and n=2 for liposomes placebo). Statistical significant differences (* P<0.05) between PDP loaded and placebo pH-sensitive liposomes. Statistical significant differences (# P<0.05) when comparing with non-modified pH-sensitive liposomes.

Hydrodynamic size and polydispersity index

The liposomes mean hydrodynamic diameter size and polydispersity index results are presented in Table 2. Generally, observing the obtained results, the mean hydrodynamic diameters, as well as, the polydispersity index values of targeted pH-sensitive liposomes were higher than the non-modified liposomal formulation.

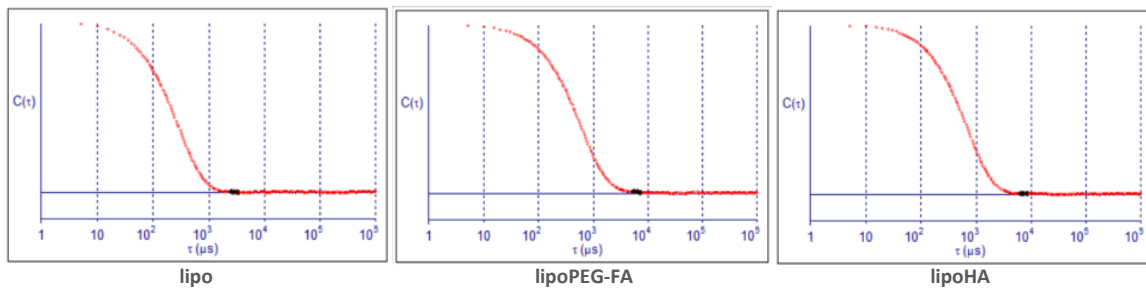


Figure 17 Correlation function plots of designed pH-sensitive liposomes.

Polydispersity is a measure of heterogeneity of a liposomal suspension, whereas polydispersity index values yield the width of the distribution of the delay times in the correlation function (Figure 17). On all the plots of designed liposomal formulations can be observed a logarithmic correlation function. Thus, indicating homogeneity of vesicles dispersion due to their uniform sizes, distinctive of LUV. Indeed, a liposomal formulation is considered monodisperse if the value of polydispersity is below 0.2. [102] By observing the obtained polydispersity index results on Table 2, it can be seen that all designed pH-sensitive liposomes presented values close to 0.1, so indicating relatively monodisperse liposomal formulations (non-aggregated vesicles) and thereby liposomal stability.

Throughout the analysis of the mean hydrodynamic diameter results on Table 2 of all designed liposomal formulations, it can be seen that liposomes modified with PEG₂₀₀₀-FA conjugate led to a small increase in vesicle size, while HA ligand showed a much higher one. This observation is valid for both liposomal formulations with and without the encapsulated drug (i.e. placebo). Thereby, it represents physical evidence that the functionalization occurred.

PEG₂₀₀₀ is a neutral, crystalline, thermoplastic biopolymer with a high solubility in water, thus is commonly used to improve the stability and biological performance of drug delivery systems. [103] In addition with the FA water solubility, the ability of the synthesized conjugate PEG₂₀₀₀-FA to prevent the self-aggregation of pH-sensitive liposomes is considered as a possible way by which it extends circulation longevity and also targetability. In fact, the water molecules forms directional bonds with PEG₂₀₀₀ such that there is an association of water molecules with a PEG₂₀₀₀ chain. The water molecules associated with PEG₂₀₀₀ create a steric hydration shell where the water molecules are oriented in a structured manner surrounding the polymer chain. [103] Therefore, the insertion of PEG₂₀₀₀-FA conjugate into liposomal membrane leads to a small increase in vesicle size, as already reported in order studies. [81, 83] Spite being small, this increase is statically significant, when compared with non-modified pH-sensitive liposomes.

When designing a pH-sensitive liposome coated with PEGylated lipids (in aqueous media), it is assumed that the PEG₂₀₀₀ will extend away from the liposome into the solvent as it is not attracted to the lipid bilayer, thus the polymer provides kinetic rather than thermodynamic protection of the liposomal surface. [103] Indeed, there are two regimes for polymers attachment to the liposome surface depending on the

molecular weight and the graft density of the polymer. If the density is low (<5 mol %) the PEG₂₀₀₀ is said to be in the mushroom regime, while if the graft density is high (>5 mol %) the PEG₂₀₀₀ are said to be in the brush regime. [103] Likewise, the degree of surface coverage and distance between graft sites on the surface of brush PEG₂₀₀₀-FA pH-sensitive liposomes was determined by the molecular weight of PEG₂₀₀₀, as well as, the 8% of graft density. However, through a more close observation to the hydrodynamic diameter results of modified pH-sensitive liposomes on Table 2, it can be observed statically significant differences ($P < 0.05$) on the decrease of the mean size of PDP loaded liposomes when compared to placebo. This is mainly due to electrostatic interactions that can be established at neutral pH (7.4) between positively charged sodium group of PDP and the terminal negatively charged groups of both FA molecules and on the head of DPPE. Since, PDP is simultaneously able to be adsorbed on the liposome surface attached to DPPE and linked to FA molecules, somehow the PEG₂₀₀₀ brush chain curl in, thereby decreasing the vesicle diameter in size. More evidences of this theory can be observed in the morphology of PEG₂₀₀₀-FA targeted liposomes assessed by TEM.

In physiological conditions, HA is in the form of a sodium salt, which confers it a highly hydrophilicity. Thus, HA is surrounded by a sphere of water molecules linked by hydrogen bonds, creating a steric hydration shell where the water molecules are oriented in a structured manner surrounding the polymer chain. [70] Therefore, namely due to the high molecular weight and strong intermolecular interactions that HA molecules perform in physiologic conditions, pH-sensitive liposomes targeted with HA present a statistical significant increase on the vesicle hydrodynamic diameter, when comparing with non-modified pH-sensitive liposomes, as can be seen in the results of Table 2 and which is consisted with previous reports. [84, 88, 104]

Zeta potential

Zeta potential is one of the main forces that mediate inter-liposomal interactions. Liposomes with a high zeta potential of the same charge sign, either positive or negative, will repel themselves and won't aggregate. Zeta potential values beneath -30 mV or above +30 mV are considered to have good liposomal shelf stability. [94]

The terminal amino group of the DPPE molecule is protonated and is thus positively charged in physiological pH (7.4), while the phosphate group is negatively

charged, which results in an overall neutral, zwitterionic, molecule. [80] Nevertheless, at pH 7.4, the terminal carboxylic group of CHEMS is negatively charged, therefore liposomes composed of DPPE:CHEMS have negative zeta potential values.

The zeta potential values of the based pH-sensitive liposomes were higher than -30 mV (Table 2). The PEG₂₀₀₀-FA modified liposomes presented a slight decrease, not statistically significant, which is in accordance with previously published results. [83]. Still, no aggregate formation occurred, even though the nature of the conjugation of the PEG₂₀₀₀ polymer to the lipid anchor may have an effect on the surface potential of the resulting PEGylated pH-sensitive liposomes. Indeed, the conjugation of PEG₂₀₀₀ to DSPE involves a carbodiimide linkage that results in a net negative charge on the phosphate group of the PEG₂₀₀₀-DSPE at physiological pH. [103] In addition, at pH 7.4, the terminal carboxylic group of FA is negatively charged, therefore liposomes consisted of DPPE:CHEMS:PEG₂₀₀₀-FA still had negative zeta potential values. Nevertheless, through a more close observation to the results on Table 2 of PEG₂₀₀₀-FA modified pH-sensitive liposomes, the zeta potential values with entrapped PDP becomes more negative than placebo, possibly due to the drug's interaction with the carboxylic group of FA. But, still not statically significant.

Moreover, results on Table 2 show that HA modified pH-sensitive liposomes provide a strong chemical binding, which lead to a sharp statically significant increase of zeta potential value, as already reported in order studies, hence resulting in a higher physicochemical stability. [84, 105] Again, in physiological conditions, HA is in the form of a sodium salt, therefore negatively charged and being able to establish strong intermolecular interactions. [70] Thus, upon addition of the PDP molecules, it establishes interactions primarily of electrostatic nature with the drugs positively charged sodium groups, and it can be observed on Table 2 statistical significant differences (* $P < 0.05$) in comparison with placebo. Nevertheless, the zeta potential negativity of designed pH-sensitive liposomes was not influenced.

5.1.3 Morphology

Morphology was assessed using TEM and representative images of each formulation of designed pH-sensitive liposomes loading PDP and placebo can be visualized on Figure 18, 19 and 20. TEM allowed a more detailed morphologic view of the liposomal formulations, observing the notable differences between samples at pH

5.0 and 7.4. In Figure 18 top images it is possible to observe liposomes composed of DPPE:CHEMS loading PDP (left) and placebo (right), respectively, in Hepes buffer at pH 7.4. These formulations presented vesicles with circle shape morphology containing populations of liposomes with homogenous sizes around 100 nm. However, it could also be observed some sob-reposition of liposomes (shadow blur), possibly due to the high concentration of the sample. Undoubtedly, the incorporation of PDP did not seem to cause morphological changes.

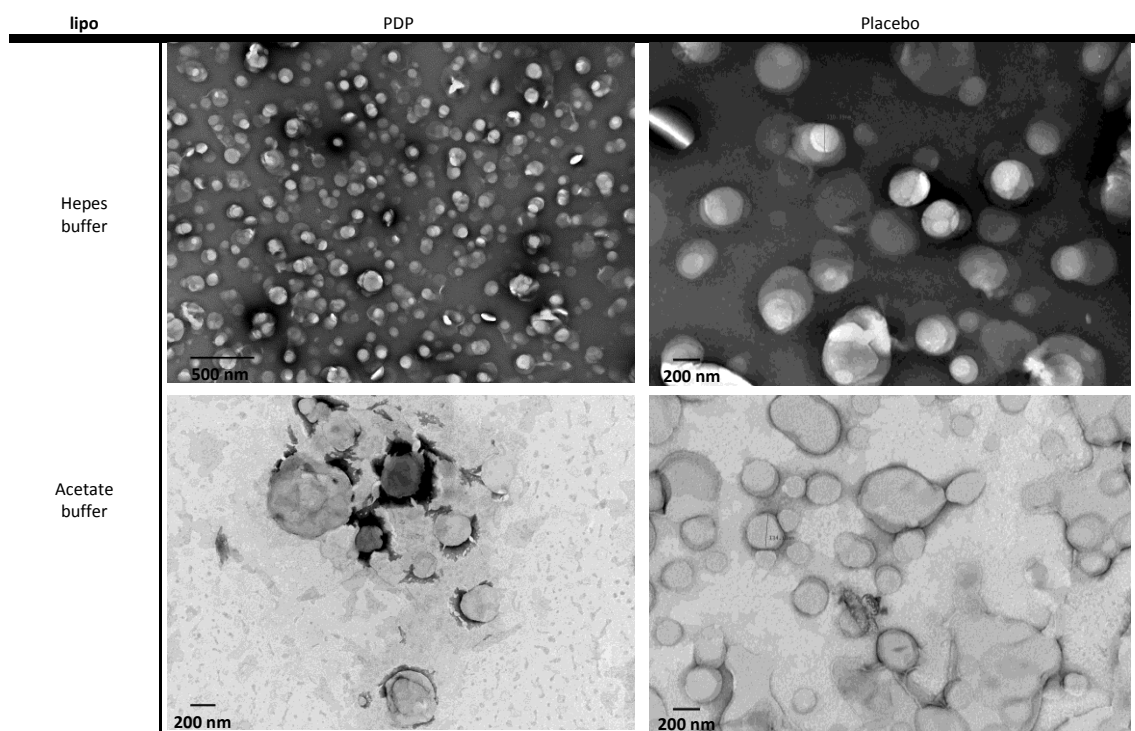


Figure 18 TEM representative images of pH-sensitive liposomes composed of DPPE:CHEMS loading PDP (left) and placebo (right) in Hepes buffer (top) and Acetate buffer (below).

Figure 18 below images are representative of liposomes composed of DPPE:CHEMS loading PDP (left) and placebo (right), respectively, in acetate buffer at pH 5.0. It is possible to observe that for both formulations at pH 5.0 there is a complete loss of structure when compared to liposomes at pH 7.4. At the acidic pH, the liposomes do not lose the spherical shape however they became much larger when compared with the ones at pH 7.4.

In Figure 19 top images it is possible to observe liposomes composed of DPPE:CHEMS:PEG₂₀₀₀-FA loading PDP (left) and placebo (right), respectively, in Hepes buffer at pH 7.4. Placebo liposomes exhibited a disc shape morphology and a white coated film is observed in the surface, whereas liposomes encapsulated with PDP morphology is spherical. Thereby, the incorporation of PDP seems to cause

morphological changes. The effects of PEG₂₀₀₀-FA on the structure of liposomes without loaded PDP was mainly due to the 8% mol of graft density, which can be related with the appearance of essentially flat bilayer discs like observed in Figure 19 (right). The appearance of bilayer discs is not expected to be stable, due to the unfavourable exposure of hydrophobic material at the edge of the discs. However, the discs were found to be stable on the time scale of weeks and so there must be a mechanism that stabilizes the discs toward fusion or closure. The possible conclusion is that the PEG₂₀₀₀-FA conjugates are situated preferentially at the highly curved edge. [106, 107]

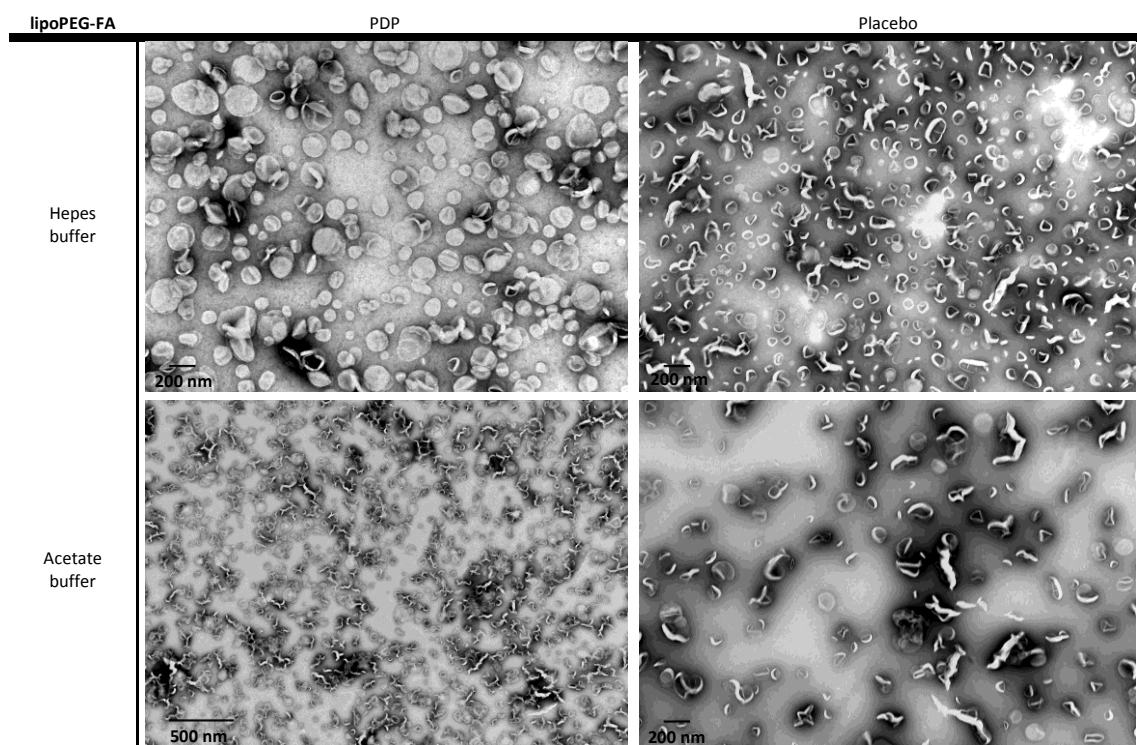


Figure 19 TEM representative images of pH-sensitive liposomes composed of DPPE:CHEMS:PEG-FA loading PDP (left) and placebo (right) in Hepes buffer (top) and Acetate buffer (below).

Moreover, note that at 8% mol of PEG₂₀₀₀-FA, the PEG₂₀₀₀ were in the so-called brush regime because the distance between the grafting points is significantly smaller than the unperturbed radius of the polymer. In this regime the PEG₂₀₀₀ overlap laterally and is, therefore, forced to stretch out into the bulk solution. Thus, the driving force for the disc formation is a relief of the energy stored in the polymer brush. When the PEG₂₀₀₀-FA conjugates are situated at the highly curved edge, the polymer-accessible volume increases (curvature effect) and, thus, confirming the results in Table 2, the increase of hydrodynamic diameter and polydispersity index values of PEG₂₀₀₀-FA targeted pH-sensitive liposomes. [106, 107]

Nevertheless, the PDP loaded pH-sensitive liposomes exhibited a sphere shape morphology, mainly due to electrostatic interactions that could be established at neutral pH (7.4) between positively charged sodium group of PDP and the terminal negatively charged phosphate groups of DPPE, hence the PDP adsorbed to the liposome surface, could also establish electrostatic interactions with FA molecules.

Figure 19 below images are representative of liposomes composed of DPPE:CHEMS:PEG₂₀₀₀-FA loading PDP (left) and placebo (right), respectively, in acetate buffer at pH 5.0. The shape morphology of liposomes, both placebo and PDP, was destroyed, mainly due to the acidic pH that provokes destabilization of the bilayer and reversion back to the inverted hexagonal phase, through the mechanism described in chapter 3. Thus, in the images can be observed the formation of lamellar fragments.

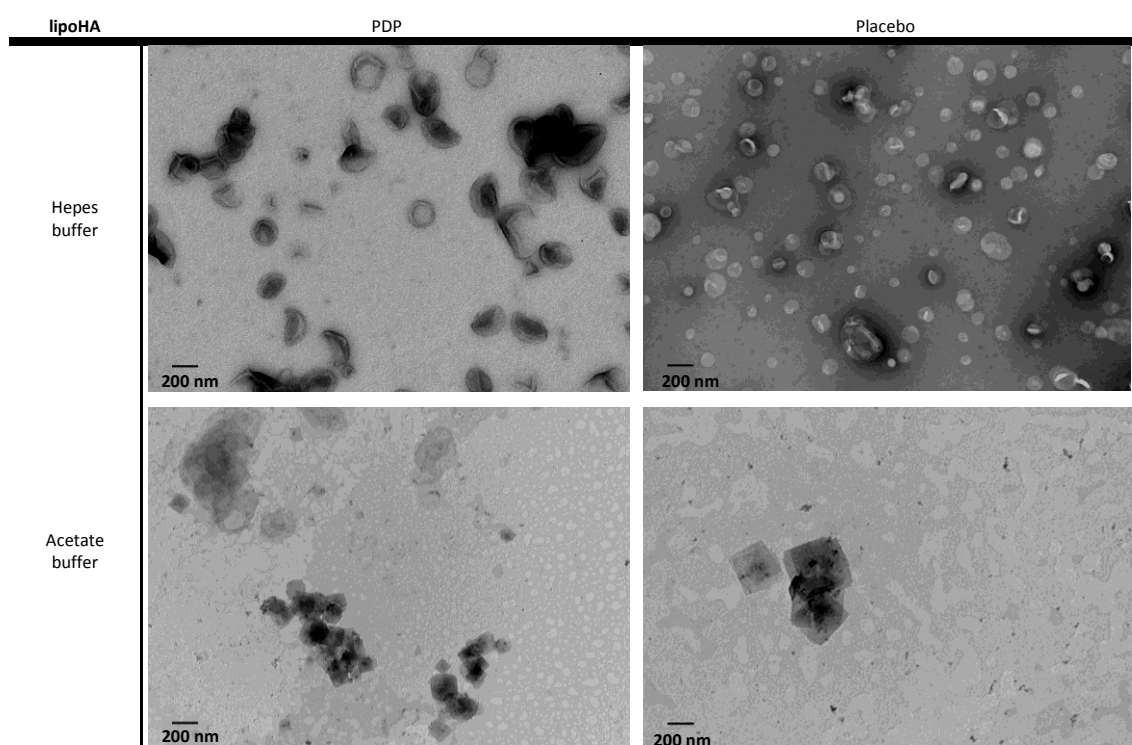


Figure 20 TEM representative images of pH-sensitive liposomes composed of DPPE:CHEMS:HA loading PDP (left) and placebo (right) in Hepes buffer (top) and Acetate buffer (below).

In Figure 20 top images it is possible to observe liposomes composed of DPPE:CHEMS:HA loading PDP (left) and placebo (right), respectively, in Hepes buffer at pH 7.4. Liposomes encapsulating PDP exhibited a ring morphology in both oval and spherical shapes, while placebo liposomes presented a sphere shape with what it's believed to be fragmented lamellar tangles of HA. Thereby, similar to PEG₂₀₀₀-FA targeted pH-sensitive liposomes, the incorporation of PDP seemed to cause morphological changes. Then again, this occurred possibly due to electrostatic

interactions established at neutral pH (7.4) between PDP adsorbed to the liposome surface and negatively charged HA molecules attached to the liposome surface. Indeed, another confirmation that support this theory was evidenced through a close observation on Table 2, as the zeta potential values of PDP loaded HA targeted pH-sensitive liposomes decreased when compared with placebo.

Figure 20 below images are representative of liposomes composed of DPPE:CHEMS:HA loading PDP (left) and placebo (right), respectively, in acetate buffer at pH 5.0. Here, it can be observed modification of the morphological shape of the liposomes when in acidic pH solution. The both PDP and placebo liposomes presented square shape and higher size, mainly due to the hexagonal phase formation triggered by the acidic pH.

5.2 Stability studies

Stability is a critical factor that must be considered during formulation design and development. Physical and chemical stability under storage conditions as well as in a biological conditions, must be considered. Thereby, all abovementioned physical characterization parameters were measured after the preparation process of all designed liposomal formulations and weekly tested for at least one month in storage conditions in terms of stability at pH 7.4 (i.e. liposomes dissolved in Hepes buffered solution) and protected from light at 4°C.

The stability of pH-sensitive liposomes composed of DPPE:CHEMS was evaluated throughout two month and Figure 21 is representative of the results obtained for %EE, %LC, vesicle hydrodynamic diameter, polydispersity index and zeta potential measurements. Results of EE and drug LC shown that until starting of the 8th week, the percentages weren't statically different over time. As well as, the same was observed for the zeta potential and polydispersity index results. On the last week the values of zeta potential were above -30, so liposomes may not had sufficient surface charge to repel themselves and aggregation phenomena may had occurred. Also, that possibility can be confirmed by the fact that pH-sensitive liposomes presented a mean polydispersity index value higher than 0.1 in comparison with the first measurement upon liposomes formation. Still, these value was just near to 0.2 and not higher, thus indicating relatively monodisperse liposomal formulations, but however with some aggregated vesicles. Therefore, the liposomal stability of this formulation might be

compromised at the end of 2 months. On the other hand, weren't observed no statically significant differences ($P>0.05$) on vesicle size diameter results, despite the diameter increase, particularly when comparing the mean diameter throughout 2 months of storage with the initial mean diameter upon liposomes preparation.

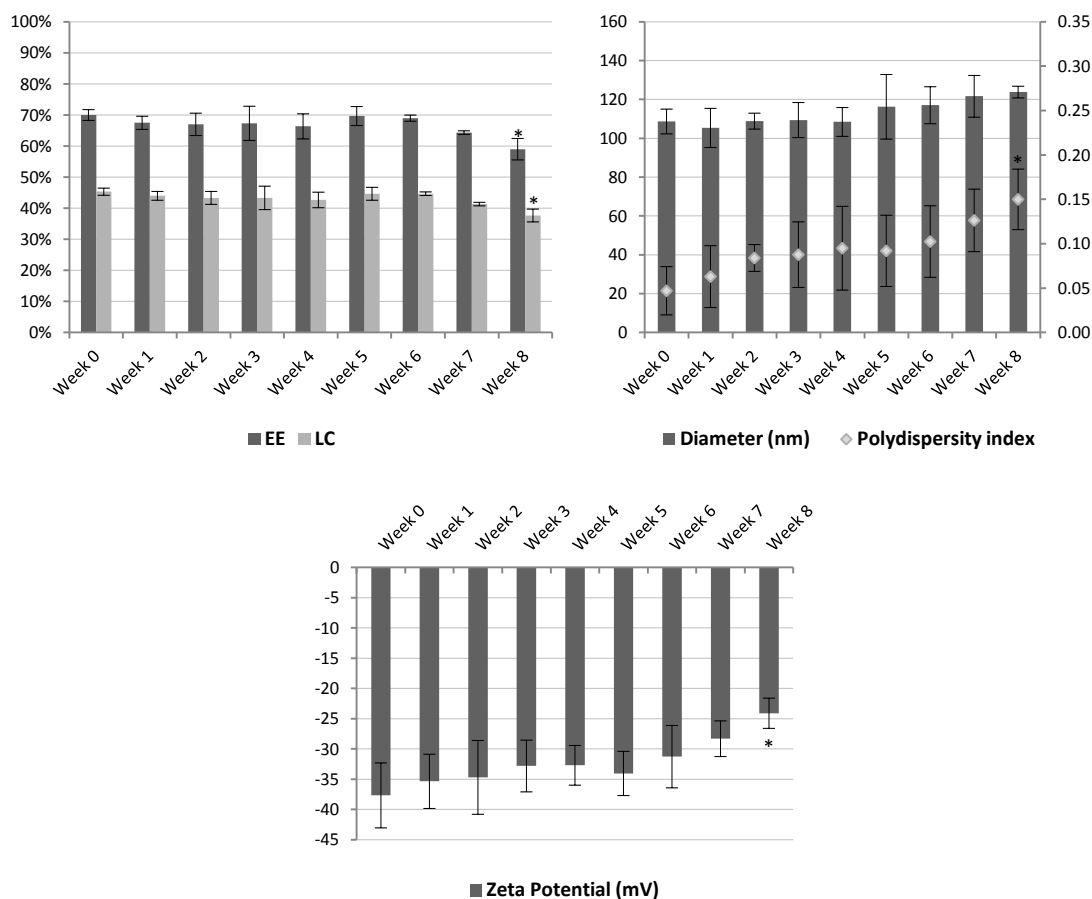


Figure 21 Effect of time of storage in stability measurements of pH-sensitive liposomes composed of DPPE:CHEMS. All data express the average \pm standard deviation ($n=3$). Statistical significant differences (* $P<0.05$).

The stability of pH-sensitive liposomes composed of DPPE:CHEMS:PEG₂₀₀₀-FA and DPPE:CHEMS:HA was evaluated throughout one month, respectively Figure 22 and 23 are representative of the results obtained for %EE, %LC, vesicle hydrodynamic diameter, polydispersity index and zeta potential measurements.

In Figure 22 it is possible to notice that the zeta potential and polydispersity results of pH-sensitive liposomes composed of DPPE:CHEMS:PEG₂₀₀₀-FA weren't statically different over time, until starting of the 3th week. On the last two weeks, the zeta potential experimental stability results of PEG₂₀₀₀-FA targeted pH-sensitive liposomes showed their tendency to be more unstable as zeta potential values are less negative than -30 mV. Thus, aggregation phenomenon was likely to occur, creating large sized

liposomal clusters. In addition, the same can be observed by the rise of polydispersity index values (proximally 0.2), since the likely existence of aggregates typically have a much broader distribution, as previously described in previous reports. [108] However, these alterations seemed not to influence the chemical stability during 1 month of the drug loaded in the targeted pH-sensitive liposomes, as there weren't observed no statically significant differences on EE of liposomes and only on the 4th week the drug LC results were significantly different from the initial measurement.

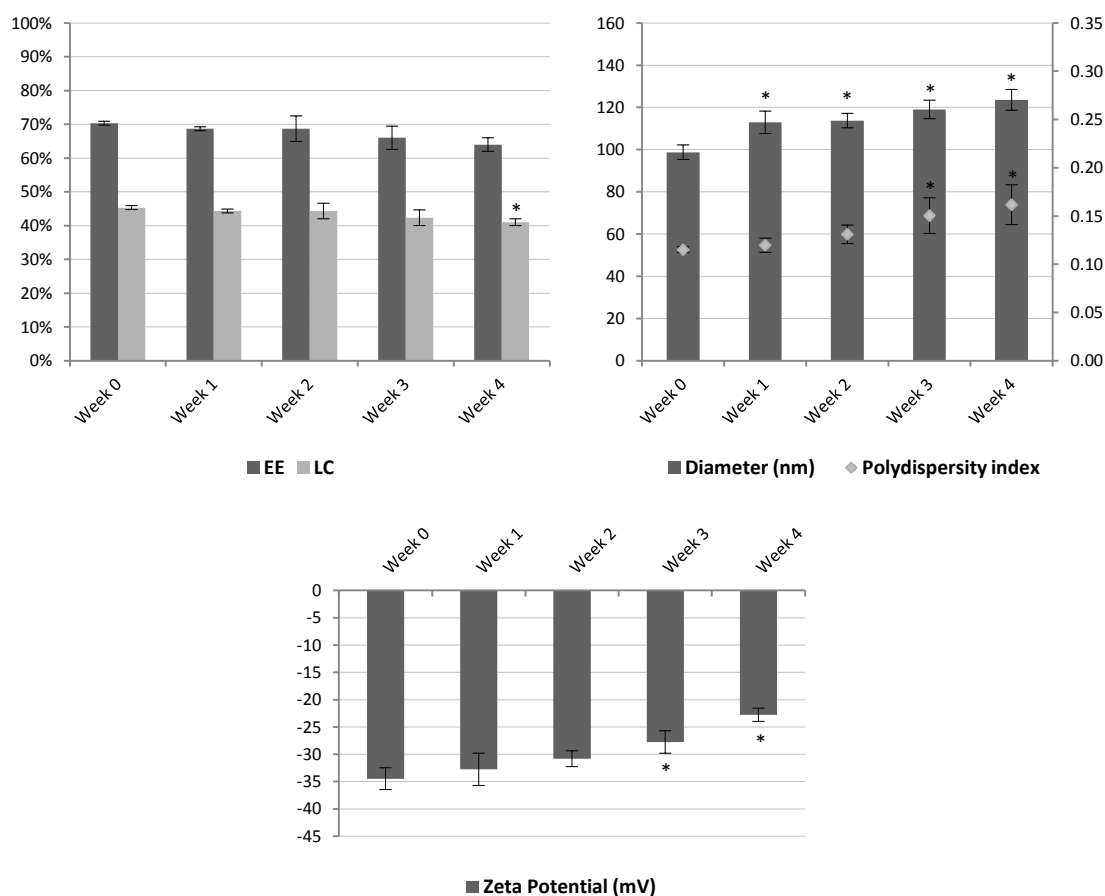


Figure 22 Effect of time of storage in stability measurements of pH-sensitive liposomes composed of DPPE:CHEMS:PEG₂₀₀₀-FA. All data express the average \pm standard deviation (n=3). Statistical significant differences (* $P < 0.05$).

Particularly, when comparing the mean hydrodynamic diameter throughout 1 month of storage with the initial mean diameter, determined upon liposomes preparation, were observed a statically significant increased on the vesicle size diameter. Still, this statistical difference can't be valid, as the first measurement was made immediately after extrusion process, hence liposomes must have had a diameter size around 100 nm. The followed weeks, by observing the diameter plot on Figure 22, the size was maintained spite of the despite the increasing on the hydrodynamic

diameter, which was mainly due, as previously explained, to the water molecules association with PEG₂₀₀₀ creating a steric hydration brush.

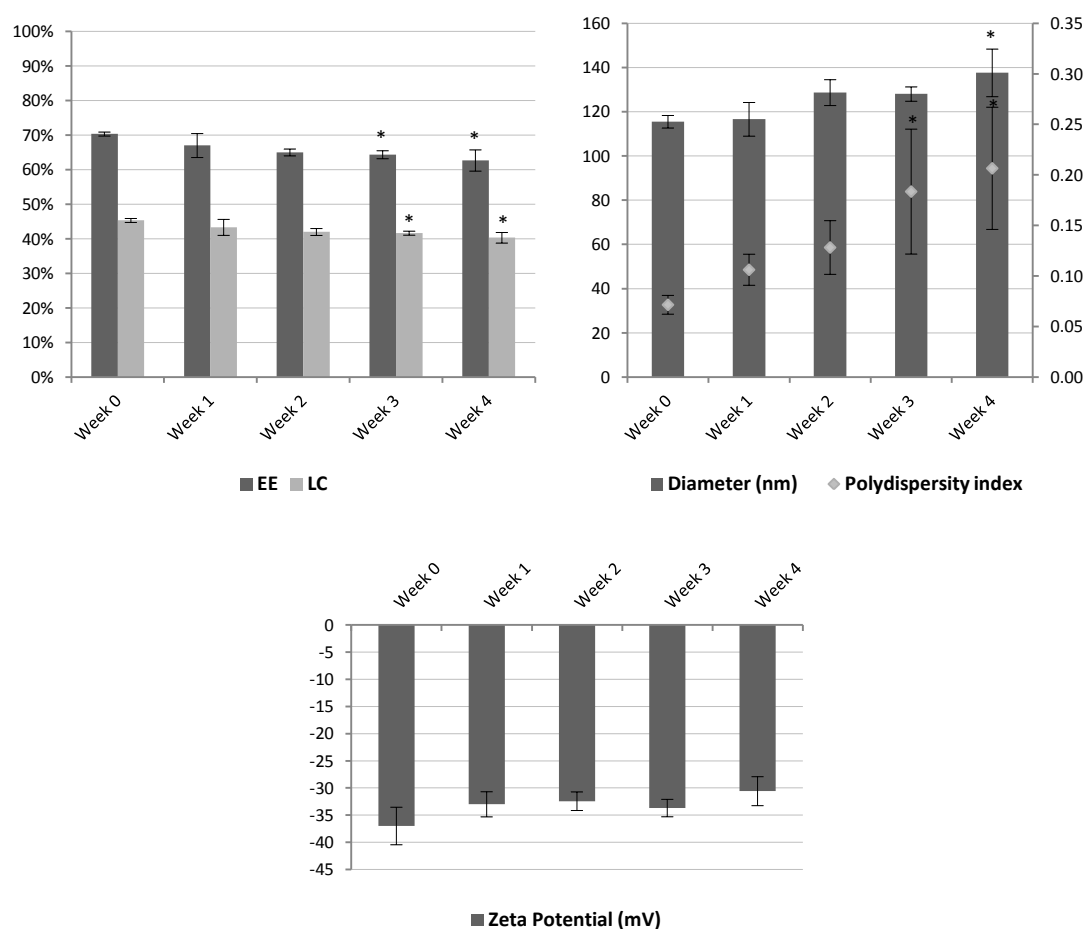


Figure 23 Effect of time of storage in stability measurements of pH-sensitive liposomes composed of DPPE:CHEMS:HA. All data express the average \pm standard deviation ($n=3$). Statistical significant differences (* $P<0.05$).

HA modified pH-sensitive liposomes are stable during 1 month, as zeta potential variations didn't appear to occur, with no statically significant differences ($P<0.05$) observed on the results presented in Figure 23. Thus, liposomes still had sufficient surface charge to repel themselves and do not aggregate. On the other hand, an increase in polydispersity index values near to 0.2 is observed, therefore the likely occurrence of aggregation phenomenon. Likewise, until the starting of the 3th week, the polydispersity results aren't statically different over time. As well as, the same statistical differences are observed for EE and drug LC percentages. Thereby, influencing the chemical stability of the drug loaded in the liposomes.

Moreover, in Figure 23 it is possible to observed that only on the 4th week the vesicle hydrodynamic diameter results of HA targeted pH-sensitive liposomes were

significantly different ($P < 0.05$), when comparing the mean diameter throughout 1 month of storage with the initial mean diameter upon liposomes preparation.

Concluding, in which concerns stability, both non and HA modified pH-sensitive liposomes seemed to be more stable than the ones modified with PEG₂₀₀₀-FA, regarding size and polydispersity index. Still, in general, it could be concluded that all the designed pH-sensitive liposomes were stable in storage conditions for at least 3 weeks, which is a huge accomplishment, as the uniform size, constant zeta potential and suitable drug loading was maintained. Nevertheless, in order to increase stability throughout time, liposomal formulations could be lyophilized. However, the optimizations of this procedure is time-consuming and due to lack of time the possibility to apply it was not possible.

5.3 Drug release studies

In vitro release studies were performed to estimate the drug release profiles of the each designed liposomal formulation over a period of 50 hours in maintained sink conditions at 37°C. Generally, the *in vitro* release profile of PDP of all designed pH-sensitive liposomes presented in Figure 24 exhibited a much higher drug release in acidic pH conditions then at physiological pH as expected. In fact, in all liposomal formulations statically significant differences ($P < 0.05$) are observed, when comparing the mean percentage of drug release from liposomes maintained in physiologic conditions (i.e. in Hepes buffer at pH 7.4) with the ones placed in acidic conditions (i.e. in Acetate buffer at pH 5.0) at each time point. Therefore, these results shown as expected the pH sensitivity of the designed liposomes as a trigger to controllably release the GC drug over time.

Furthermore, generally observing all the drug release profiles in Figure 24, liposomes at pH 7.4 steady 30% of the amount of PDP released during 20 hours, while at acidic pH it occurred in half the time. Indeed, the percentage of drug release over time at physiologic pH (what was not supposed to happen) was proximally correspondent to the unloaded drug.

Through a closer observation of each liposomal formulation, it could be concluded that the fast release was found for modified pH-sensitive liposomes (Figure 24B and C), whilst the slower release was found for the non-modified liposomal formulation (Figure 24A). Such fact, suggest that the major fraction of drug was entrapped into the

polymeric network of HA or PEG₂₀₀₀ and/or adsorbed onto the liposomal surface rather than into the liposome.

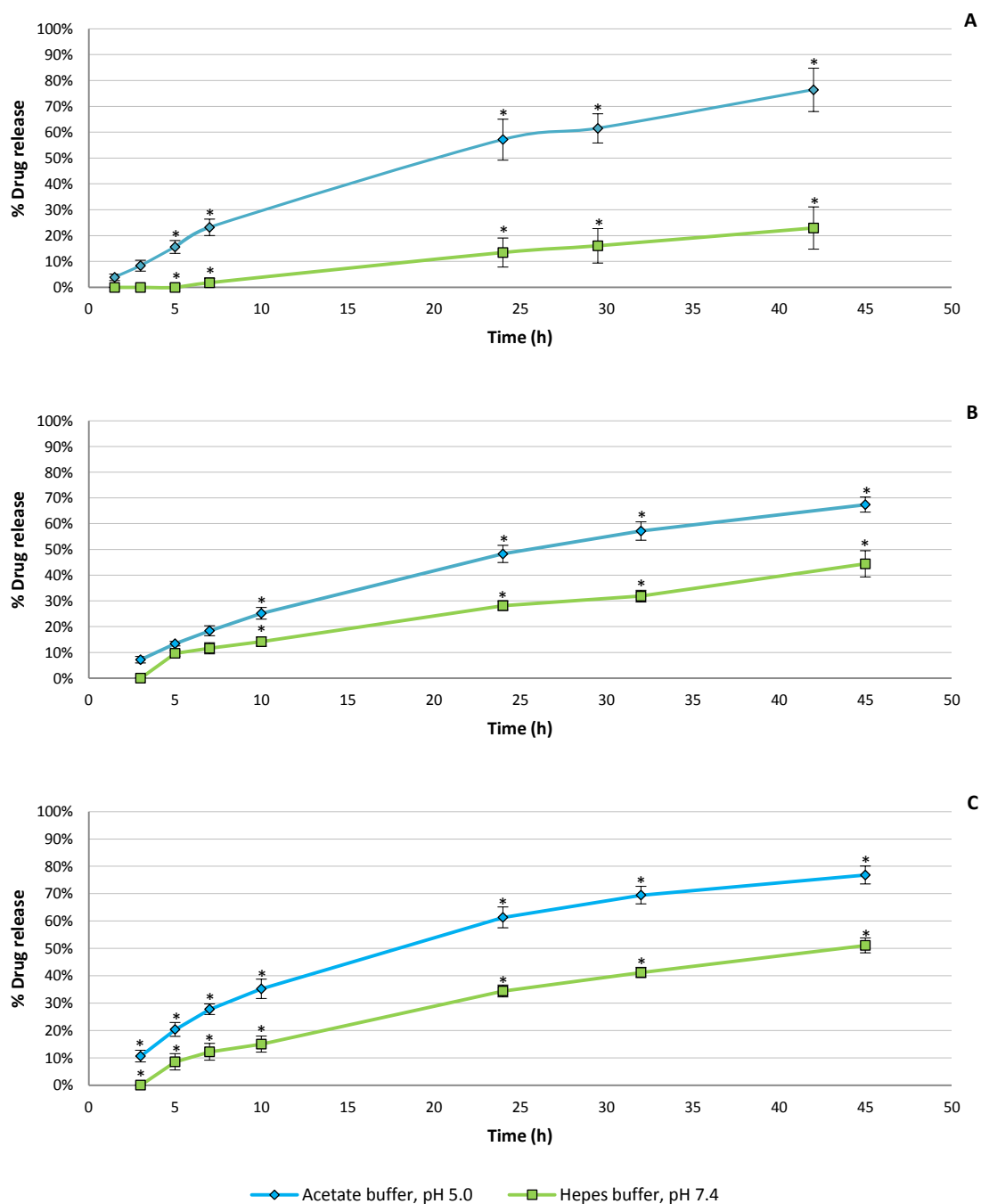


Figure 24 *In vitro* release profiles of PDP from pH-sensitive liposomes (A) modified with PEG₂₀₀₀-FA (B) or HA (C) in both acidic and physiological conditions at 37°C. All data express the average \pm standard deviation (n=3). Statistical significant differences (* $P < 0.05$) between Hepes and Acetate buffers at each time point.

Furthermore, comparing the percentage of drug release in Acetate buffer at time point 35h of each formulation release profile, it can be observed that FA-PEG₂₀₀₀ modified pH-sensitive liposomes presented the lowest release. Thereby, suggesting, as

reported in order studies, that the inclusion of DSPE-PEG₂₀₀₀-FA conjugate in DPPE:CHEMS liposomes decreases their pH-sensitivity due to stabilization of the bilayer by PEG₂₀₀₀ molecules. However, it has been shown that when these liposomes interact with cells, the intracellular release of their contents is not affected, which is also confirmed on followed cellular studies, and therefore the inclusion of the conjugate does not decrease the efficiency of designed pH-sensitive liposomes. [37, 109]

Nevertheless, these results suggest that a suitable amount of PDP loaded in designed pH-sensitive liposomes would be stable in the blood circulation and when reached the target cell, in endosomes at acidic conditions at least 50% of the drug loaded will be for sure controllably release, as the acidic pH provokes destabilization of the liposome bilayer and reversion to the inverted hexagonal phase.

5.4 Calcein study

Fluorescence quenching

The percentage of fluorescence quenching depends on the ratio between DPPE and CHEMS. The used molar ratio between DPPE and CHEMS of 6.5:3.5, as had already been described in previous studies, liposomes with this constitution and ratio exhibit high pH-sensitivity and form also tightly closed stable vesicles.

The percentage of quenching of fluorescence depends on the ratio of DPPE to CHEMS. The molar ratio of DPPE to CHEMS was 6.5:3.5, as had already been described in previous studies, liposomes exhibit high pH-sensitivity and form also tightly closed stable vesicles. [37] The quenching % of calcein fluorescence was calculated using equation (4) and was found to be 56%, which it is a relatively acceptable value, since the quenching value of PE is very low, as they readily form hexagonal phases instead of a liposomal bilayer without a complementary molecule such as CHEMS. By this means that the liposomal membranes with low quenching value are unstable and then somehow they destabilize during the gel permeation chromatography process, or the liposomal membrane is so loose that an appreciable amount of entrapped calcein is released. [53, 110] Therefore, it should be taken into account the amount of CHEMS, in order to obtain stable liposomes, necessary if this study wants to be performed.

pH-dependent calcein release

To design pH-sensitive liposomes with optimal release characteristics, it is necessary to evaluate the liposomal membrane integrity as a function of pH and, likewise, the bilayer permeability upon exposition to PDP solution. The calcein release profiles from pH-sensitive liposomes presented in Figure 25 show that when pH-sensitive liposomes encapsulating a self-quenching calcein concentration were exposed to an acidic environment (Acetate buffer, pH 5.0), the calcein was released due to destabilization of the liposomal bilayer and diluted in the surrounding media, resulting in a fluorescence intensity signal of 93%. This result is in good agreement with previous reports. [37, 53]

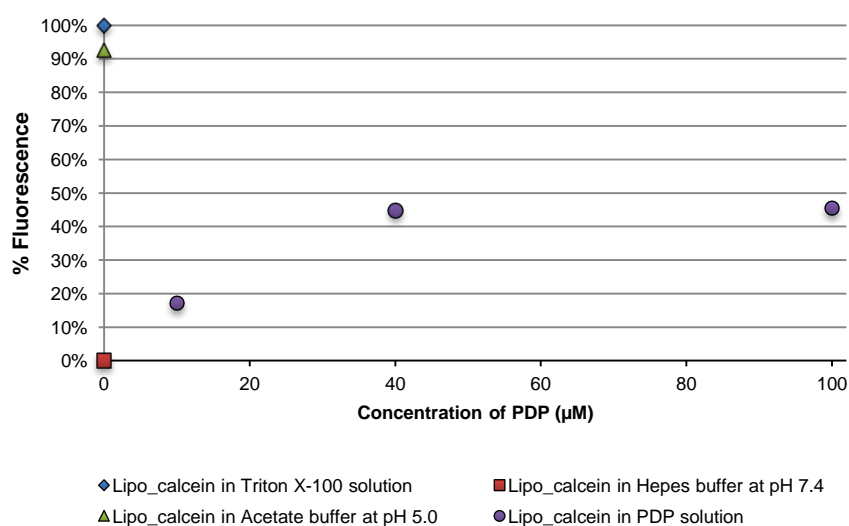


Figure 25 Calcein release profiles from pH-sensitive liposomes.

Moreover, in Figure 25, it can be observed that the exposition of calcein loaded pH-sensitive liposomes to 10, 40, 100 μM of PDP solution, influenced the liposomal membrane permeability. Results show that the fluorescence intensity arising from calcein release increases with the increase of PDP concentration, up to 45%. Therefore, despite the concentration of PDP increases, liposomes are not able to permeate more than 40 μM when competing with calcein, being therefore saturate.

5.5 Cellular studies

The cytosolic liposomal delivery of PDP was evaluated in both macrophage RAW 264.3 and fibroblast L929 murine cell lines by comparing the cell viability and

cytotoxicity of designed pH-sensitive liposomes loading PDP and free PDP. Placebo pH-sensitive liposomes in addition to free PDP were also included as a control, in order to evaluate the possibility of inherent liposomal cytotoxicity. Thus, the following sub chapters present the results of MTT viability, LDH cytotoxicity and uptake assays that were carried out in both RAW and L929 cell lines.

5.5.1 L929 cell viability and cytotoxicity

The MTT and LDH assay results in L929 fibroblasts presented on Figure 26 show that, as expected, the formulation of pH-sensitive liposomes composed of DPPE:CHEMS:HA was the most cytotoxic in this cell line, as result of a higher release of LDH enzyme and, as well as, a lower cell metabolic activity.

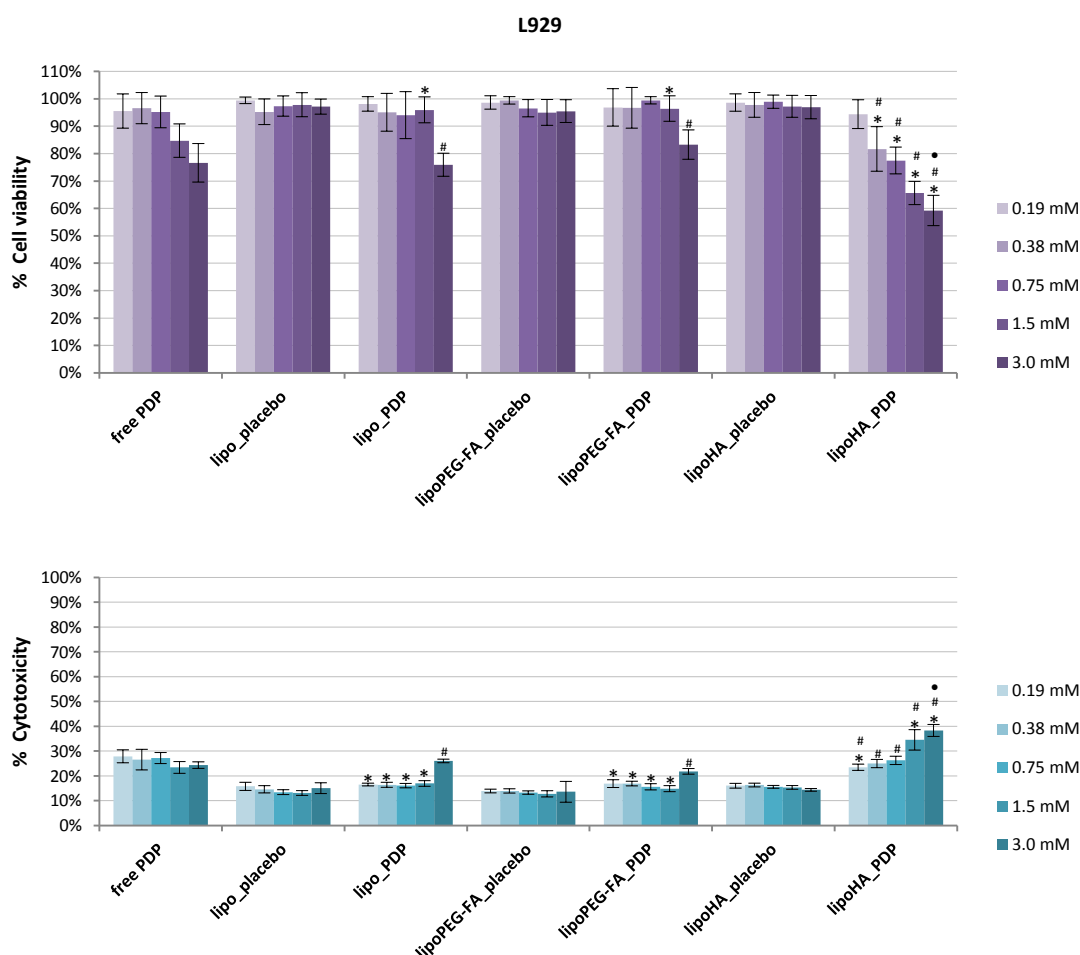


Figure 26 L929 viability (up) and cytotoxicity (down) of free PDP and all designed pH-sensitive liposomes at a range of concentrations (0.19 – 3.0 mM). All data express the average \pm standard deviation (n=5 of two independent assays). Statistical significant differences when comparing designed pH-sensitive liposomes loading PDP) with free PDP (* $P < 0.05$) and with placebos (# $P < 0.05$) at each concentration. Statistical significant differences of pH-sensitive liposomes with the targeted ones (• $P < 0.05$) at the highest concentration.

In fact, significant statistical differences ($P < 0.05$) at higher concentrations (1.5 and 3.0 mM) of liposomal formulations on both assays were observed, when comparing with free PDP and placebo controls. In Figure 26, it can be observed that only at the highest concentration a slight reduction on metabolic activity (MTT) exists and a not statistically significant cytotoxicity, when compared with free PDP, for both nonmodified and targeted PEG₂₀₀₀-FA pH-sensitive liposomes.

Nevertheless, as expected, the placebos corresponding to each designed liposomal formulation did not influence significantly the membrane permeability (LDH), therefore being biocompatible with L929 fibroblast. Therefore, the resulted cytotoxicity has to be due an enhanced permeation of PDP, which increases its cellular concentration and leads to enhanced cytotoxicity. Likewise, this means that the chosen targeting molecule for the fibroblasts cell line is efficient and allows an enhanced cellular uptake.

5.5.2 RAW cell viability and cytotoxicity

The results of MTT and LDH assays in RAW macrophages presented on Figure 27, show that both targeted pH-sensitive liposomes loaded with PDP had a higher cytotoxic effect than with non-modified pH-sensitive liposomes, as result of a lower cell metabolic activity and higher release of LDH enzyme. In fact, significant statistical differences ($P < 0.05$) were observed on both assays at higher concentrations (1.5 and 3.0 mM), when comparing with free PDP and respective placebo controls. In the case of non-modified pH-sensitive liposomes, only at the highest concentration the metabolic activity was slightly and the membrane permeability influenced.

Placebos of each designed liposomal formulation showed to be biocompatible with macrophages, unless for PEG₂₀₀₀-FA modified pH-sensitive liposomes. This liposomal formulation was not cytotoxic in L929 cell line. Nevertheless, in RAW macrophages a decrease of mitochondrial activity (MTT) of about 40% for the highest concentration was observed. Despite that, when loaded with PDP an even stronger reduction in mitochondrial activity of about 65% was observed. Thereby, the main resulted cytotoxicity is due to the effect of the drug, once it as an increased cellular concentration, result of an enhanced permeation through the cellular membrane.

Although, PEG₂₀₀₀-FA modified pH-sensitive liposomes had an efficient cytotoxic effect in RAW macrophages (Figure 27), contrary to what was expected, RAW cells

appeared to be more sensitive than L929 cells when incubated with HA modified pH-sensitive liposomes, which conjugate was the ultimate active targeting to synovial fibroblast. Thereby, MTT and LDH assays should be performed in an environment that better mimics the inflamed synovium. By this means, culture RAW cell line in free folate RPMI-1640 medium, which is unique from DMEM medium because it not contains the FA vitamin, thus cells would be able to over express *in vitro* high amounts of FA receptor. Then, theoretically, enhance cellular internalization of PEG₂₀₀₀-FA modified pH-sensitive liposomes, which results in increased cytotoxicity.

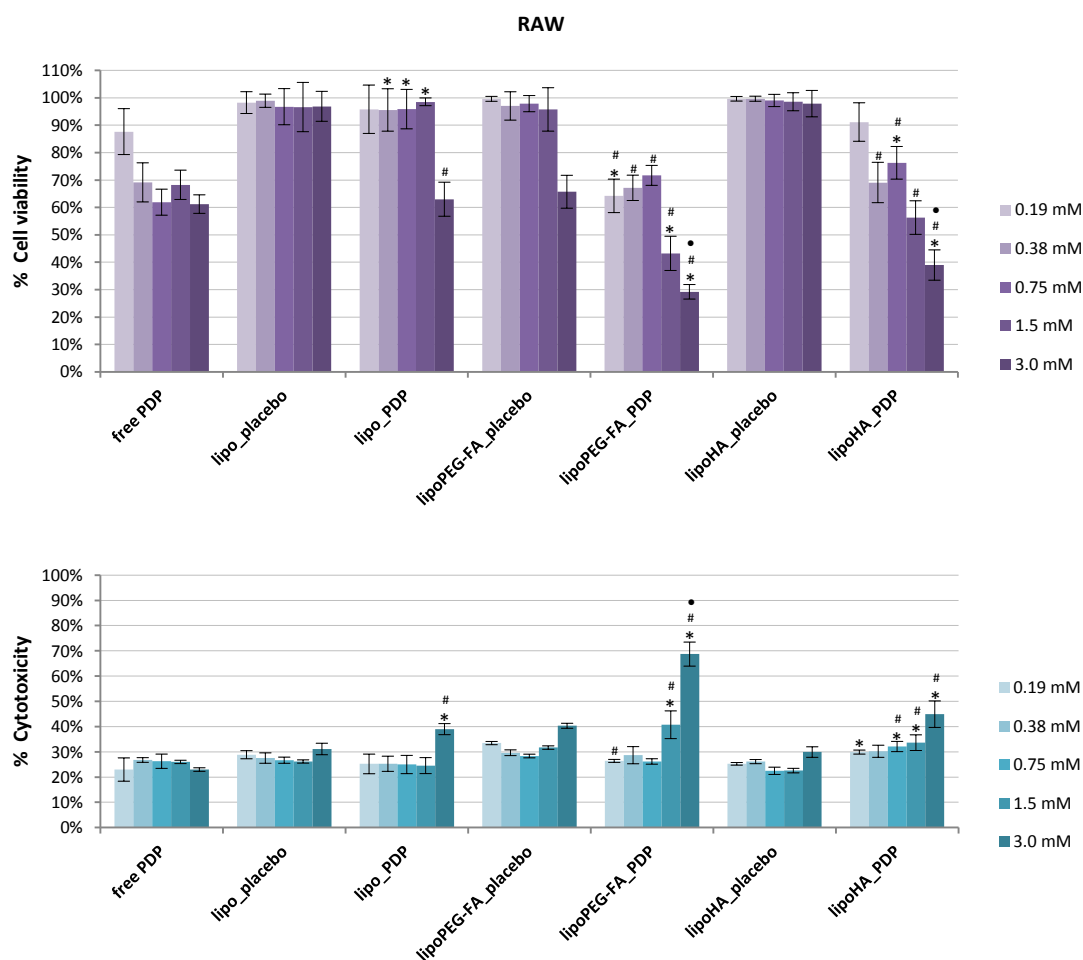


Figure 27 RAW viability (up) and cytotoxicity (down) of free PDP and all designed pH-sensitive liposomes at a range of concentrations (0.19 - 3.0 mM). All data express the average \pm standard deviation (n=5 of two independent assays). Statistical significant differences when comparing designed pH-sensitive liposomes loading PDP with free PDP (* $P < 0.05$) and with placebos (# $P < 0.05$) at each concentration. Statistical significant differences of pH-sensitive liposomes with the targeted ones (• $P < 0.05$) at the highest concentration.

Half maximal inhibitory concentration

The half maximal inhibitory concentration (IC_{50}) for free PDP and PDP loaded liposomal formulations were compared for cytotoxicity based on the MTT assay of both RAW and L929 cell lines. Table 3 summarizes the IC_{50} values obtained after 24 hours incubation with free PDP and designed pH-sensitive liposomes in a concentration range of 0.19 to 3.0 mM.

Table 3 IC_{50} values of free PDP and designed liposomal formulations for both RAW and L929 cell lines.

IC 50	Free	Lipo	LipoPEG-FA	LipoHA	Lipo	LipoPEG-FA	LipoHA
	PDP				Placebo		
RAW	-	-	1.32 mM	2.03 mM	-	-	-
L929	-	-	-	-	-	-	-

Throughout the IC_{50} values presented in Table 3, it can be shown that it was not possible to determine the IC_{50} for free PDP in both cell lines. In order to overcome the out of concentration range, additional *in vitro* studies should be performed using a wide concentration range higher than 3.0 mM. Similarly, since none of the designed liposomal formulations without PDP present cytotoxicity in both RAW and L929 cell lines, being therefore biocompatible, it was not possible to define the IC_{50} value. In fact, it was only for RAW macrophage cell line that the IC_{50} value was defined for PDP targeted pH-sensitive liposomes. In the range of concentration evaluated, PEG₂₀₀₀-FA modified pH-sensitive liposomes achieved an IC_{50} value at 1.32 mM, meaning that, as expected, this liposomal formulation enhanced the accumulation of drug on macrophages, hence increasing drug cytotoxicity. Still, HA modified pH-sensitive liposomes presented some cytotoxicity (IC_{50} = 2.03 mM) on RAW macrophages, spite being needed a higher concentration of liposomal formulation.

5.5.2 Cell uptake

The intracellular uptake kinetics of designed pH-sensitive liposomes was assessed in both cell lines, RAW and L929, and detected by fluorescence intensity following various incubation times, as presented in Figure 28. The volume of liposomes (400 μ L) used corresponds to a lipid concentration of 1 mM and a conjugated fluorescein NBD equivalent concentration 1 % mol, respectively, and does not significantly affect the

viability of the cells at this concentration. For the measurement of the intracellular amount of designed pH-sensitive liposomes by flow cytometry, the mean fluorescence intensity of untreated cells (controls) measured 3032 for RAW and 7048 for L929. Washing after cell incubation with PBS was performed to guarantee the removal of liposomes located on the cell surface and only measurement of fluorescence intensity coming from internalized liposomes.

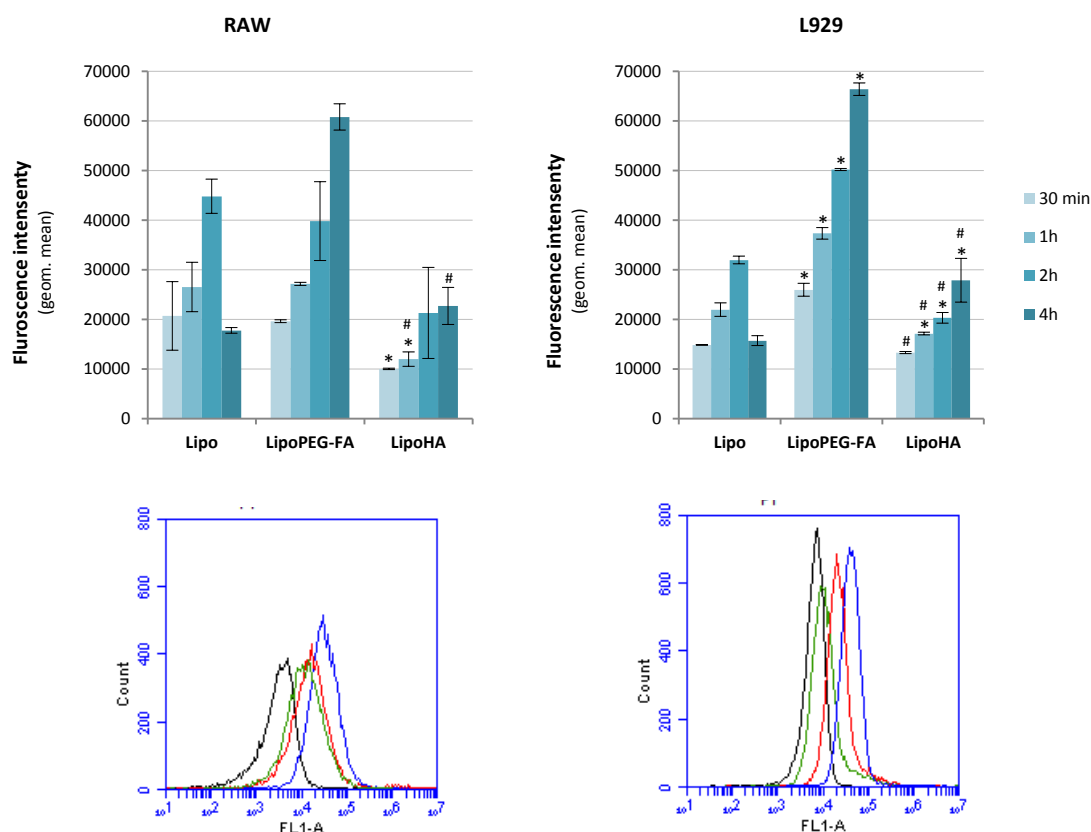


Figure 28 Cellular uptake on RAW and L929 of designed pH-sensitive liposomes labeled with FL1 (excitation wavelength of 488 nm), as determined by flow cytometry. Respective obtained histograms at 2h of incubation time of non-modified (green) and PEG₂₀₀₀-FA (blue) or HA (red) modified pH-sensitive liposomes, plus cell controls (black). All data express the average \pm standard deviation (n=2). Statistical significant differences when comparing non-modified pH-sensitive liposomes with targeted ones (* $P < 0.05$) at each incubation time. Statistical significant differences when comparing PEG₂₀₀₀-FA and HA modified pH-sensitive liposomes (# $P < 0.05$) at each incubation time.

As shown in Figure 28, cell penetration of pH-sensitive liposomes occurs, and a significant internalization was detected as early as 30 min after treatment at 37 °C, since high fluorescence intensity values could be measured within the cells, implying a high uptake during this short incubation period, which demonstrates that designed pH-sensitive liposomes translocate rapidly into the target cells.

A pronounced increase in uptake can be observed for PEG₂₀₀₀-FA modified pH-sensitive liposomes after 4 h in both RAW macrophages and L929 fibroblast, thus this

liposomal formulation had the more efficient uptake effect than with non-modified or HA modified pH-sensitive liposomes. In fact, no significant difference ($P < 0.05$) was observed for HA modified pH-sensitive liposomes associated fluorescence between 2 h and 4 h of incubation in RAW cells, indicating that the uptake is almost complete within 2 h.

Although, PEG₂₀₀₀-FA modified pH-sensitive liposomes had an efficient uptake effect in both cell lines (Figure 28), contrary to what was expected, the fluorescence intensity results in L929 cells appeared to be higher than in RAW cells. Thereby, this assay should also be performed in an environment that better mimics the inflamed synovium by incubating RAW cell line in free folate RPMI-1640 medium, to enhance the *in vitro* over-express of FA cell receptor. Hence, theoretically, increasing the uptake of PEG₂₀₀₀-FA modified pH-sensitive liposomes by RAW macrophages. In addition, the increased uptake of designed pH-sensitive liposomes for RAW cells during the initial accumulation can be attributed to the macrophage ability to internalize invader vesicles from 50 to 300 nm either by clathrin-mediated endocytosis, phagocytosis, macropinocytosis, caveolae-mediated endocytosis or non-clathrin-non-caveolae-dependent endocytosis. [111] On the other hand, fibroblasts do not have internalization mechanisms as developed as macrophage, which may explain the results obtained in the uptake assay and likewise upon IC₅₀ determination.

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Chapter 6

Conclusion

RA is a chronic and progressive autoimmune disease that is characterized by inflammation of the synovial tissue of joints and destruction of cartilage hence bone erosion. The disease pathology is complex, involving cellular immune activation and along with the release of pro-inflammatory mediators and cytokines into the pannus of affected joints, the proliferation of synovial cells. The conventional GC treatment is used in 30 to 60% of patients for joint disease. Still, GCs like PDP are hampered by their highly unfavorable pharmacokinetic associated with severe toxic side effects, limited selectivity and widespread biodistribution of drug molecules into non-targeted tissues. Hence, low drug bioavailability on target cells and thus a decreased therapeutic efficiency. The main goal of designed targeted pH-sensitive liposomes was to overcome these drawbacks, enhancing a selective and efficient delivery of PDP into target synovial macrophages and fibroblast. In this sense, pH-sensitive liposomes composed of 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) and cholesteryl hemisuccinate (CHEMS), or DPPE:CHEMS:PEG-FA or DPPE:CHEMS:HA (respectively, in a molar ratio of 6.5:3.5, 6.5:3.5:0.08, 6.5:3.5:0.03) were successfully developed, by thin film hydration technique followed by extrusion, and physicochemically characterized in terms of hydrodynamic size, polydispersity, zeta potential, encapsulation efficiency, drug loading capacity and morphology assessed using TEM. All the designed liposomal formulations show to be relatively uniform in size (~ 100 nm) with a low polydispersity index (~0.1) and a zeta potential bellow - 30 mV. The average drug encapsulation efficiency and loading capacity were respectively 70% and 45%. Thereby, a suitable amount of drug for therapeutic efficiency is loaded in the pH-sensitive liposomes. In addition, regarding all parameters of physicochemical

characterization, stable liposomal formulations were achieved for at least a period of 1 month at 4°C.

The *in vitro* drug release studies from pH-sensitive liposomes mimicking both biological conditions at pH 7.4 and pH 5.0 exhibited, as expected, a higher release in acidic mimic biological conditions than at physiological pH.

Moreover, *in vitro* cellular studies using RAW 264.7 and L929 cell lines, respectively, of macrophage and fibroblasts, were performed to evaluate both cell viability and cytotoxicity character of designed liposomal formulations. Regarding the MTT and LDH assays, it is possible to realize that designed targeted pH-sensitive liposomes specifically enhanced the accumulation of PDP on both macrophages and fibroblast, then improving drug cytotoxic effects, when compared with free PDP or non-modified pH-sensitive liposomes. Likely, due to the FA-FR β and HA-CD44 receptors mediated endocytosis of targeted pH-sensitive liposomes (Figure 29), which resulted on the efficient intracellular delivery of PDP, hence allowing a higher drug bioavailability into synovial cells. Similarly, cellular uptake kinetic studies of designed pH-sensitive liposomes through the target synovial cells assessed by flow cytometry, suggest that HA and FA targeting ligands would enhance the cellular uptake of liposomally entrapped PDP, thus improving a high drug bioavailability and hence its therapeutic efficiency.

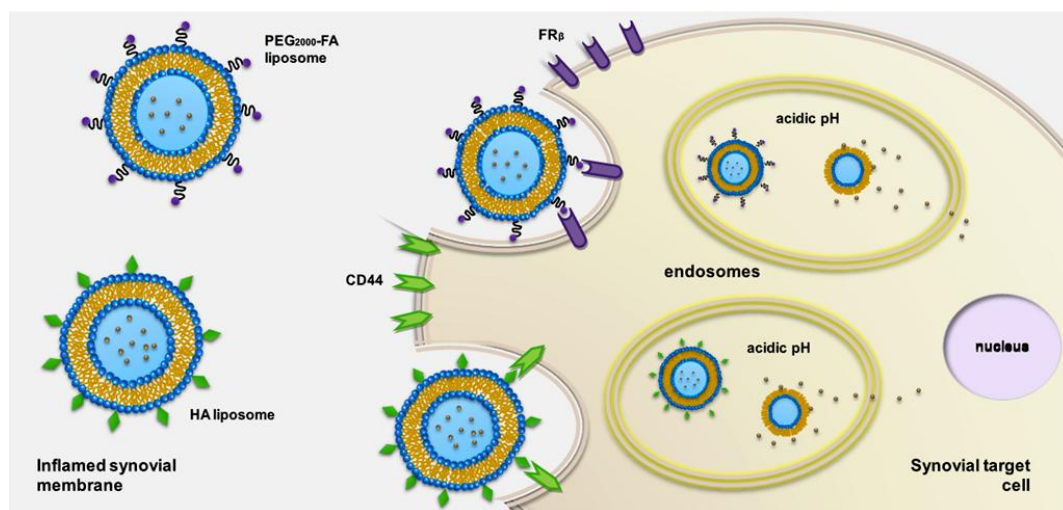


Figure 29 Receptor mediated endocytosis of design targeted pH-sensitive liposomes.

The therapeutic potential of designed pH-sensitive liposomes was increased by the optimization of liposomes physicochemical parameters, with particular emphasis on the size, the zeta potential, the phospholipids nature, the morphology of the vesicles, the quantity of loaded drug molecules and the binding kinetics of liposomes to target

cell surface receptors by means of specific targeting ligands. Since these characteristics influenced the stability and *in vitro* performance of the designed liposomes, upon liposome-cell interactions allowing a selective internalization into target cells. And ultimately, the pH-sensitivity mechanism trigger the control release of loaded PDP, hence increasing its bioavailability. In conclusion, the design and characteristics of pH-sensitive liposomes targeted with PEG₂₀₀₀-FA or HA enhance the therapeutic efficiency of PDP in RA treatment.

Further remarks

Designed pH-sensitive liposomes have already presented great potential as drug delivery systems to selectively enhance the therapeutic efficiency of PDP in RA treatment. However, there are always several *in vitro* and, as well as, *in vivo* studies, that can be performed for further optimization of the liposomal formulation.

- *In Vitro* Studies
 - Structural characterization of targeted pH-sensitive liposomes assessed by nuclear magnetic resonance (NMR) spectroscopy, in order to quantify the functional groups of targeting ligands;
 - MTT and LDH assays with RAW and L929 to achieve statistical significance;
 - MTT and LDH assays with RAW and L929 cultured in free folate RPMI-1640 medium;
 - MTT and LDH assays with human cells, taken from synovial tissue of patients diagnosed with and without RA (collaboration with Santo António Hospital);
 - Uptake kinetics assay assessed by flow cytometry, with RAW and L929 cultured in free folate RPMI-1640 medium;
 - Validation in an induced-inflammation assay assed by flow cytometry;
 - Mechanisms of uptake and intracellular location fluorescent imaging assessed by confocal microscopy.
- *In Vivo* Studies
 - Efficacy, biodistribution and toxicity assays in animal model (healthy vs arthritic) after intravenous injection of designed pH-sensitive liposomes.

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